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Lipoxygenase metabolites of arachidonic and linoleic acids modulate the adhesion of tumor cells to endothelium via regulation of protein kinase C

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12(S)-hydroxyeicosatetraenoic acid (12[S]-HETE) and 13(S)-hydroxyoctadecadienoic acid (13[S]-HODE), lipoxygenase metabolites of arachidonic acid and linoleic acid, respectively, previously have been suggested to regulate tumor cell adhesion to endothelium during metastasis. Adhesion of rat Walker carcinosarcoma (W256) cells to a rat endothelial cell monolayer was enhanced after treatment with 12(S)-HETE and this 12(S)-HETE enhanced adhesion was blocked by 13(S)-HODE. Protein kinase inhibitors, staurosporine, calphostin C, and 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, inhibited the 12(S)-HETE enhanced W256 cell adhesion. Depleting W256 cells of protein kinase C (PKC) with phorbol 12-myristate-13-acetate abolished their ability to respond to 12(S)-HETE. Treatment of W256 cells with 12(S)-HETE induced a 100% increase in membrane-associated PKC activity whereas 13(S)-HODE inhibited the effect of 12(S)-HETE on PKC translocation. High-performance liquid chromatographic analysis revealed that in W256 cells 12-HETE and 13-HODE were two of the major lipoxygenase metabilites of arachidonic acid and linoleic acid, respectively. Therefore, these two metabolites may provide an alternative signaling pathway for the regulation of PKC. Further, these findings suggest that the regulation of tumor cell adhesion to endothelium by 12(S)-HETE and 13(S)-HODE may be a PKC-dependent process.

Introduction

Tumor cell metastasis is a multistep process involving homotypic and heterotypic interactions among tumor cells and host cells, in addition to tumor cell adhesion to matrix proteins (Weiss *et al.,* 1989). These interactions are mediated by a variety of cell surface receptors, including the integrins (Hynes, 1987) and cell–cell adhesion molecules (Takeichi, 1990). The factors that regulate membrane expression/activation of these receptors may be critical determinants of the metastatic phenotype. These regulatory factors can be intracellular effectors of signaling pathways or extracellular effectors such as cytokines, growth factors, etc. The tumor promoter phorbol 12-myristate-13-acetate (PMA)[1] induces cellular responses including the increased expression of integrin receptors on tumor cells resulting in their enhanced adhesion to endothelium and subendothelial matrix (Honn *et al.,* 1988; Grossi *et al.,* 1989). The mechanism responsible for these effects of PMA are believed to be activation of protein kinase C (PKC) because PMA is one of the most potent activators of that kinase. PKC plays an important role in the signal transduction process that modulates a variety of cellular responses, including secretion, contractility, migration, and growth (Nishizuka, 1984; O'Brian and Ward, 1989). Induction by PMA of PKC activation and/or translocation of PKC to membrane increases the metastatic ability of some tumor cells (Gopalakrishina and Barsky, 1988; Korczak *et al.,* 1989), suggesting that PKC-mediated signal transduction mechanisms are involved in the metastatic cascade.

Arachidonic acid is metabolized by prostaglandin endoperoxide synthase to prostaglan-

[1] Abbreviations used: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; H7, 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine; H8, *N*-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide; HBSS, Hank's balanced saline solution; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; mAb, monoclonal antibody; MEM, Eagle's minimal essential medium; PMA, phorbol 12-myristate-13-acetate; PKC, protein kinase C; RAEC, rat aortic endothelial cell; RP-HPLC, reverse phase-high performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; W256 cells, Walker 256 carcinosarcoma cells.

dins and thromboxanes and by lipoxygenases to monohydroxy fatty acids. It was observed that a lipoxygenase metabolite of arachidonic acid, 12(S)-hydroxyeicosatetraenoic acid (12[S]-HETE), produced by normal cells as well as tumor cells (Buchanan and Bastida, 1987; Marnett *et al.*, 1991), mimics the effect of PMA on tumor cell adhesion (Grossi *et al.*, 1989). In contrast, a lipoxygenase metabolite of linoleic acid, 13(S)-hydroxyoctadecadienoic acid [13(S)-HODE], which is produced by endothelial cells and leukocytes as well as tumor cells (Buchanan and Bastida, 1987), antagonizes the effects of 12(S)-HETE on both the increased surface expression of the integrin receptor complex αIIbβ3 and the enhancement of tumor cell adhesion to endothelium and subendothelial matrix (Grossi *et al.*, 1989). However, the mechanism of action of 12(S)-HETE- and/or 13(S)-HODE-induced alterations in tumor cell adhesion is unknown. Arachidonic and linoleic acids, as well as some of their metabolites, have been shown to directly activate PKC exhibiting moderate effectiveness in high micromolar concentrations (Hansson *et al.*, 1986; Holian *et al.*, 1989; Fan *et al.*, 1990). However, the cellular responses elicited by 12(S)-HETE and 13(S)-HODE are observed at high nanomolar concentrations (Honn *et al.*, 1988; Grossi *et al.*, 1989), suggesting the possibility of an alternative mechanism for PKC activation by these hydroxyfatty acids.

Therefore, we examined the involvement of PKC in the heterotypic adhesion of tumor cells to endothelium as modulated by lipoxygenase metabolites of arachidonic and linoleic acids. In this study, we provide evidence that 1) 12(S)-HETE enhances W256 cell adhesion to endothelium whereas 13(S)-HODE inhibits this enhanced adhesion, 2) the stimulated adhesion of tumor cells to endothelium involves a process mediated by PKC, and 3) 12(S)-HETE induces membrane translocation of PKC in W256 cells whereas 13(S)-HODE antagonizes this effect. We propose a signaling pathway for PKC activation/inactivation involving lipoxygenase metabolites of arachidonic acid and linoleic acid. Further, we suggest that modulation of PKC by certain lipoxygenase metabolites of arachidonic and linoleic acids may be a key event in the regulation of tumor cell adhesion to endothelium.

Results

12(S)-HETE enhances adhesion of W256 cells to rat aortic endothelial cells (RAEC)

The effect of 12(S)-HETE on adhesion to RAEC was examined by treating W256 cells with various concentrations of 12(S)-HETE (from 20 to 2000 pmol/10^6 cells) for 3 min or with 200 pmol/10^6 cells for timed intervals from 1 to 15 min. Upon treatment with 12(S)-HETE, an enhancement of W256 cell adhesion to RAEC was observed in a dose- and time-dependent manner (Figure 1, A and B). Maximal enhancement (>100%) was observed after a 3-min treatment with 200 pmol/10^6 cells (Figure 1, A and B). Treatment with doses of 12(S)-HETE > 200 pmol/10^6 cells or for longer time intervals resulted in smaller enhancement of W256 cell adhesion to RAEC. Similar dose- and time-dependent effects have been observed for 12(S)-HETE enhanced B16 amelanotic melanoma cell adhesion to murine pulmonary endothelium (Liu, unpublished data). The effect of 12(S)-HETE on W256 cell adhesion to RAEC was specific because neither 5(S)-HETE, 11(S)-HETE, 12(R)-HETE, nor 15(S)-HETE treatment (3 min, 37°C) at concentrations ranging from 20 to 2000 pmol/10^6 cells induced any significant enhancement in adhesion (Figure 2).

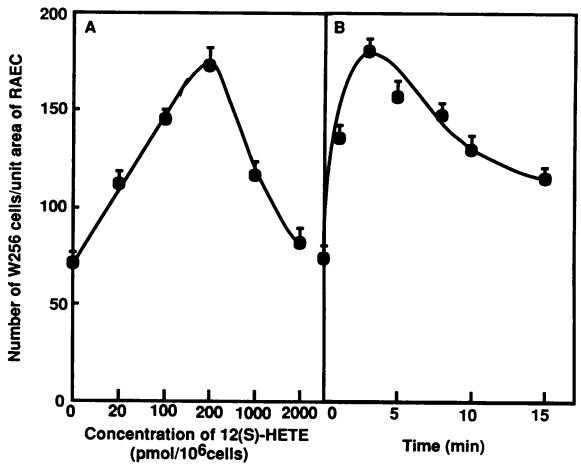

Figure 1. **12(S)-HETE-enhanced W256 cell adhesion to RAEC.** (A) Dose-response of 12(S)-HETE-enhanced W256 cell adhesion. W256 cells were treated with indicated concentrations of 12(S)-HETE for 3 min at 37°C. Final ethanol content during treatment of tumor cells was kept constant (0.1%) in control cells and 12(S)-HETE-treated cells. When working at the higher concentration of the dose-response curve for 12(S)-HETE, a measured volume of stock 12(S)-HETE dissolved in absolute ethanol was dried on ice under a gentle stream of nitrogen gas in reduced light and reconstituted in a smaller volume of absolute ethanol so that the final ethanol content was always 0.1%. (B) Time dependence for 12(S)-HETE-enhanced W256 cell adhesion. W256 cells were treated with 200 pmol/10^6 cells of 12(S)-HETE for indicated time intervals at 37°C. Adhesion assays for treated W256 cells were performed as described in Materials and methods.

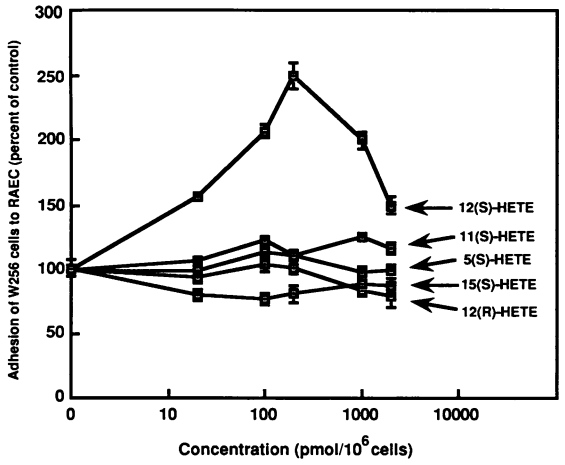

Figure 2. **Effect of other monohydroxy fatty acids derived from arachidonic acid on adhesion of W256 cells to RAEC.** W256 cells were treated (3 min, 37°C) with 5(S)-, 11(S)-, 12(R)-, or 15(S)-HETE at concentrations ranging from 20 to 2000 pmol/10^6 cells. 12(S)-HETE was included in this experiment for comparison. Ethanol content during treatment of tumor cells was kept constant (0.1%) as described in Figure 1. Adhesion assays were performed as described in Materials and methods. Results are expressed as percent of control.

13(S)-HODE inhibits 12(S)-HETE-enhanced W256 cell adhesion to RAEC

W256 cells were pretreated with either 9(S)-HODE, 13(R)-HODE, or 13(S)-HODE (200 pmol/10^6 cells, 10 min) before stimulation (3 min) with an equal concentration of 12(S)-HETE. 13(S)-HODE completely inhibited 12(S)-HETE-enhanced adhesion of W256 cells to RAEC, whereas both 13(R)-HODE and 9(S)-HODE were ineffective (Figure 3). Adhesion of cells treated with 13(S)-, 13(R)-, or 9(S)-HODE alone did not differ from controls (Figure 3).

Effect of protein kinase inhibitors on 12(S)-HETE-enhanced tumor cell adhesion to endothelium

Protein kinase inhibitors staurosporine, calphostin C, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) and *N*-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide (H8), and sphingosine (Matsui *et al.*, 1986; Tamaoki *et al.*, 1986; Kobayashi *et al.*, 1989; Smal and Meyts, 1989) were used to study the involvement of PKC in the adhesion of tumor cells to endothelium. W256 cells were pretreated (10 min) with the inhibitors followed by treatment with 12(S)-HETE (200 pmol/10^6 cells, 3 min). Protein kinase inhibitors alone did not affect W256 cell adhesion to RAEC (data not shown). However, a dose-dependent inhibition of 12(S)-HETE-enhanced adhesion was observed with each individual inhibitor exhibiting a different potency profile (Figure 4). Sphingosine and H8 were less effective at inhibiting 12(S)-HETE-induced adhesion than the potent protein kinase inhibitor staurosporine and the more specific PKC inhibitors H7 and calphostin C, which completely abolished the 12(S)-HETE effect (Figure 3).

Effect of PKC down-regulation on 12(S)-HETE-enhanced adhesion to RAEC

In addition to protein kinase inhibitors, down-regulation of PKC by chronic pre-exposure of cells to PMA has often been used to study the involvement of PKC in stimulator-induced cellular responses (Pillay *et al.*, 1990). In our study, long-term PMA treatment alone did not affect basal W256 cell adhesion to RAEC (Figure 5B). However, down-regulation of PKC by PMA completely abolished the 12(S)-HETE enhanced adhesion (Figure 5B) whereas cells treated with diluent ethanol for 24 h retained their responsiveness to 12(S)-HETE stimulation (Figure 5A). PKC activity assays confirmed that total PKC activity was decreased (39% of control) after PMA treatment. Cell viability (Trypan blue exclusion) after PMA treatment was >85%.

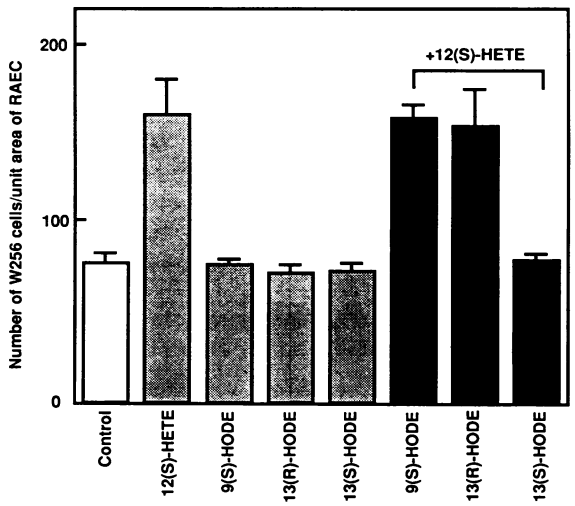

Figure 3. **Effect of linoleic acid-derived monohydroxy fatty acids on 12(S)-HETE enhanced W256 cell adhesion to RAEC.** W256 cells were preincubated (10 min, 37°C) with 9(S)-HODE, 13(R)-HODE, or 13(S)-HODE (200 pmol/10^6 cells) before treatment (3 min) with an equivalent amount of 12(S)-HETE. Control cells were treated with identical amounts of ethanol for identical time intervals. Adhesion assays were performed as described in Materials and methods.

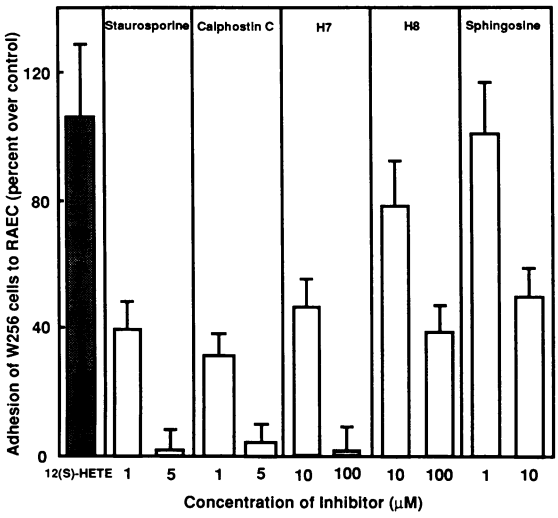

Figure 4. **Effect of protein kinase inhibitors on 12(S)-HETE-enhanced W256 cell adhesion to RAEC.** W256 cells were pretreated (10 min, 37°C) with inhibitors at indicated concentrations followed by treatment with 12(S)-HETE (200 pmol/10^6 cells, 3 min). Adhesion assays for treated cells were performed as described in Materials and methods. Results are expressed as percentage over control that was 70 ± 12 (number of W256 cells/unit area of RAEC). The inhibitors alone did not significantly enhance or decrease W256 adhesion to RAEC ($p > 0.05$ compared with control).

12(S)-HETE induces translocation of PKC in W256 cells

When W256 cells were treated with doses of 12(S)-HETE from 40 to 1000 pmol/10^6 cells for 5 min or with 200 pmol of 12(S)-HETE/10^6 cells for 1, 5, 10, or 15 min, a translocation of PKC from the cytosolic form to a membrane-bound form was observed in a dose- and time-dependent pattern (Figure 6, A and B). A 100% increase in membrane-bound PKC activity was observed after a 5 min-treatment with 200 pmol/10^6 cells (Figure 6, A and B). The bell shape of the time course and dose-response curves suggested that the effect of 12(S)-HETE on PKC translocation was transient and that down-regulation of PKC may be induced at higher concentrations. The effect of 12(S)-HETE on PKC translocation was specific because neither 5(S)-HETE, 11(S)-HETE, 12(R)-HETE, nor 15(S)-HETE induced any significant increase in membrane-associated PKC activity (Figure 7A). Total PKC activity did not change significantly after treatment with any of the HETEs.

13(S)-HODE inhibits 12(S)-HETE-induced PKC translocation

In adhesion assays, we observed that 13(S)-HODE specifically inhibited 12(S)-HETE-enhanced W256 cell adhesion to RAEC. To determine the effects of 13(S)-HODE on translocation of PKC and especially on 12(S)-HETE-induced PKC translocation, W256 cells were pretreated with 13(S)-HODE (200 pmol/10^6 cells, 10 min) followed by treatment with an equivalent amount of 12(S)-HETE (5 min). 13(S)-HODE completely inhibited the effect of 12(S)-HETE on PKC translocation (Figure 7B). Under the same conditions, 13(R)-HODE and 9(S)-HODE failed to inhibit 12(S)-HETE-induced PKC translocation. 9(S)-HODE, 13(S)-HODE, and 13(R)-HODE alone had no effect on translocation of PKC (Figure 7B).

W256 cells synthesize 12-HETE and 13-HODE

Reverse phase-high-performance liquid chromatographic (RP-HPLC) analysis demonstrated that W256 cells were capable of converting arachidonic acid and linoleic acid to 12-HETE and 13-HODE, respectively. Both 12-HETE and 13-HODE were found only in the cell extract and no detectable lipoxygenase metabolites were released into the media (data not shown). When W256 cells were incubated with 9 μM arachidonic acid for 2 min, two major metabolites coeluted with authentic 15(S)-HETE and 12(S)-

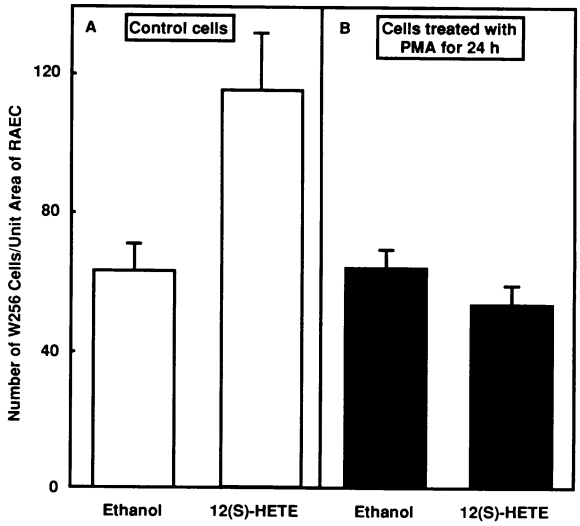

Figure 5. **Effect of PKC down-regulation on 12(S)-HETE-enhanced W256 adhesion to RAEC.** Adherent W256 cells were treated with diluent ethanol as control (A) or 0.1 μM PMA (B) in MEM at 37°C for 24 h. Cells were then suspended in MEM and further stimulated with 12(S)-HETE (200 pmol/10^6 cells, 3 min, 37°C) or diluent ethanol (control) before assay for adhesion to endothelium as described in Materials and methods.

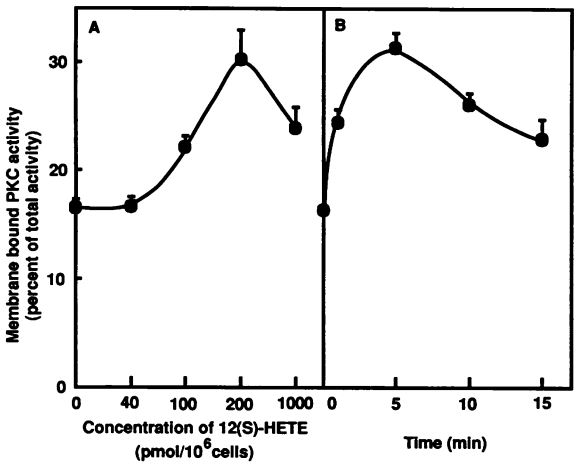

Figure 6. **12(S)-HETE-induced membrane PKC translocation in W256 cells in a dose- and time-dependent manner.** (A) W256 cells (2×10^7) were treated at 37°C with indicated amounts of 12(S)-HETE for 5 min. Final ethanol content during treatment of tumor cells was kept constant (0.1%) in control and 12(S)-HETE treated cells as described in Figure 1. (B) W256 cells were treated with 200 pmol of 12(S)-HETE/10^6 cells for indicated time intervals. PKC activities in cytosolic and membrane fractions were assayed as described in Materials and methods. Membrane-bound PKC activity was expressed as a percentage of total activity (cytosol + membrane). Total activity did not change significantly ($p > 0.05$ compared with control) after treatment with 12(S)-HETE. Total PKC activity averaged 350 pmoles of phosphate transferred · mg protein^{-1} · min^{-1}. Data represent results of three experiments performed in triplicate.

HETE (Figure 8A). Similar metabolite profiles were observed after incubation for time intervals longer than 2 min; however, the size of both peaks decreased with time. By 30 min, cells incubated with arachidonic acid showed trace amounts of lipoxygenase metabolites. The time dependence of linoleic acid metabolism was similar to that of arachidonic acid metabolism. The greatest amount of metabolites was observed after incubating W256 cells with 10 μM linoleic acid for 5 min. 13-HODE was the major product and 9-HODE the minor product (Figure 8B). The stereospecificity (i.e., S or R) of these arachidonic acid and linoleic acid metabolites was not assigned because only the S enatiomers (e.g., 12[S]-HETE and 13[S]-HODE) were biologically active (Figure 2, 3, and 7).

Immunocytochemistry of PKC in adherent W256 cells

Slot blot analysis demonstrated that the monoclonal antibody (mAb1.9) raised against rat brain PKC recognizes PKC in W256 cells and in rat brain cytosol that was used as a positive control (data not shown). Therefore, this antibody was used to localize PKC in adherent W256 cells where PKC was detected at the apical plasma membrane but not at membrane areas in contact with substratum (Figure 9a). Treatment (5 min, 37°C) of W256 cells with increasing concentrations of 12(S)-HETE (25 to 250 pmol/10^6 cells) altered the distribution of PKC in a dose-dependent manner. At a dose of 250 pmol/10^6 cells, we observed increased labeling for PKC at the apical plasma membrane. In addition, labeling for PKC was observed in the filopodia and at membrane areas in contact with the substratum (Figure 9c). A similar pattern for the altered distribution of PKC was observed over the entire dose range tested. Pretreatment of W256 cells with 13(S)-HODE (250 pmol/10^6 cells, 10 min) before 12(S)-HETE stimulation (250 pmol/10^6 cells, 5 min) did not alter the apical, filopodia, or basal membrane labeling for PKC (Figure 9e). In contrast, 13(S)-HODE pretreatment (250 pmol/10^6 cells) effectively prevented 12(S)-HETE-induced PKC translocation to both the apical plasma membrane and to areas in contact with the substratum (Figure 9g) such that the PKC distribution was comparable with untreated controls (Figure 9a). The negative control samples demonstrated no background

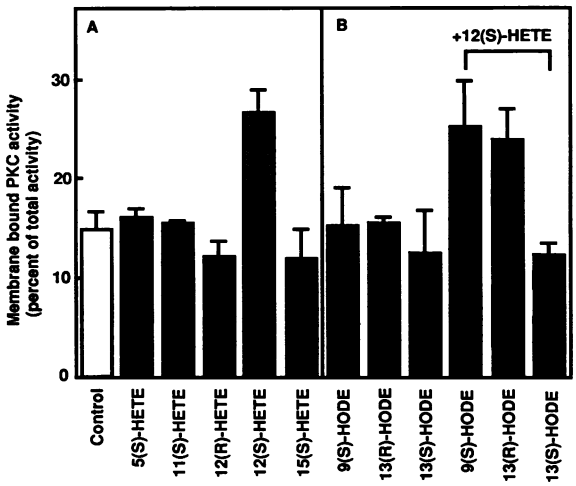

Figure 7. **Effect of monohydroxy fatty acids on translocation of PKC.** (A) W256 cells were treated (5 min, 37°C) with indicated compounds at a concentration of 200 pmol/10^6 cells. (B) W256 cells were treated (200 pmol/10^6 cells, 10 min, 37°C) with 9(S)-HODE, 13(S)-HODE, or 13(R)-HODE followed by treatment (5 min) with an equivalent amount of 12(S)-HETE. Ethanol content was kept constant (0.1%) at all treatment conditions. PKC activities in cytosolic and membrane fractions were assayed as described in Materials and methods. Values represent results of two experiments performed in triplicate.

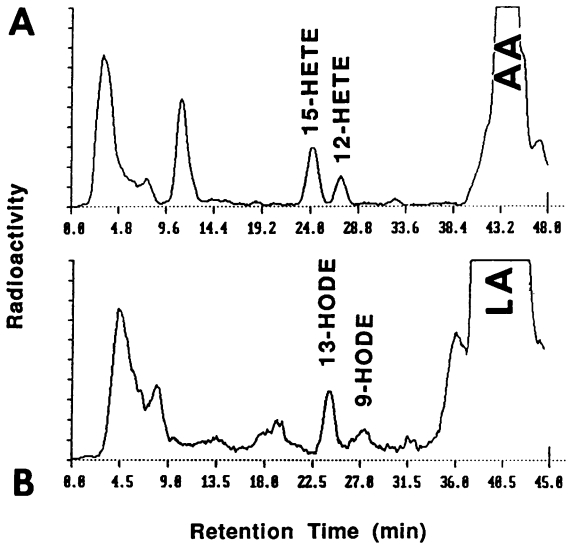

Figure 8. **Reverse phase-high-performance liquid chromatographic profiles of lipoxygenase metabolites of radiolabeled arachidonic and linoleic acids in W256 cell extract.** Cells (10^7) were incubated in MEM at 37°C with 9 μM [^{14}C]arachidonic acid for 2 min or with 10 μM [^{14}C]linoleic acid for 5 min. Cell lipids were extracted and analyzed as described in Materials and methods. (A) 15-HETE and 12-HETE peaks coeluted with corresponding anthentic compounds. The identity of 12-HETE peak was verified by addition of authentic [^{3}H]12(S)-HETE to the biologically derived sample (data not shown). AA, arachidonic acid. (B) 13-HODE and 9-HODE peaks coeluted with corresponding authentic compounds. LA, linoleic acid.

fluorescence (data not shown), confirming the specificity of the results.

Discussion

Activation of PKC in tumor cells enhances characteristics of the metastatic phenotype, such as the stimulation of lung colonizing ability (Gopalakrishina and Barsky, 1988; Korczak *et al.*, 1989). However, the actual step(s) of the metastatic cascade that is affected is unknown. During the hematogenous phase of the metastatic process, adhesion of tumor cells to the endothelium and subendothelial matrix may be rate limiting (Weiss *et al.*, 1989). Tumor cell interaction with the vessel wall is comprised of three consecutive steps: adhesion of tumor cells to endothelium, induction of endothelial cell retraction by tumor cells, and tumor cell adhesion to and spreading on the subendothelial matrix (Nicolson, 1982). This initial interaction of tumor cells with the vessel wall is followed by migration through the subendothelial matrix by a proteolytically dependent process. Since PMA stimulation of tumor cells results in increased adhesion to endothelium (Grossi *et al.*, 1989), the initial interaction of stimulated tumor cells with endothelium seems to involve PKC activation. The results presented in the present study with W256 cells and in a previous study with Lewis lung carcinoma cells (Grossi *et al.*, 1989) clearly indicate that 12(S)-HETE treatment of tumor cells results in increased adhesion to endothelium. This enhancement is mediated by activation of tumor cell PKC as shown by the inhibitory effects of PKC inhibitors (H7 and especially calphostin C) and the inhibitory effect of PKC down-regulation. Interestingly, the unstimulated basal adhesion of W256 cells to RAEC does not appear to involve PKC because it is not affected by PKC inhibitors and by down-regulation of PKC. Therefore, the interaction of tumor cells with endothelium can be divided into at least two phases: an initial attachment phase that is PKC independent and an adhesion phase that is PKC dependent. Recently, it has been demonstrated that tumor cell migration through basement membrane involves PKC activation whereas initial attachment to basement membrane is independent of PKC (Schwartz *et al.*, 1990).

12(S)-HETE is the major lipoxygenase metabolite of arachidonic acid synthesized by Lewis lung carcinoma cells (Marnett *et al.*, 1991) and one of the major metabolites in W256 cells (this study). It is also synthesized by a wide range of normal cells (Spector *et al.*, 1988). We demonstrated that among 5(S)-, 11(S)-, 12(S)-, 12(R)-, and 15(S)-HETEs only 12(S)-HETE was capable of enhancing W256 cell adhesion to endothelial cells. Similar results have been reported for Lewis lung carcinoma cells (Grossi *et al.*, 1989). However, 12(S)-HETE may not be the only effector mediating this heterotypic cell–cell interaction because our data demonstrated that 12(S)-HETE enhanced adhesion was inhibited by 13(S)-HODE. This latter monohydroxy fatty acid is a lipoxygenase metabolite of linoleic acid. In W256 cells, it is the major metabolite of linoleic acid (this study). 13(S)-HODE was originally identified as a platelet chemorepellant factor produced by endothelial cells (Buchanan *et al.*, 1985). Later studies demonstrated that some tumor cells produce 13(S)-HODE and that the ratio of 13(S)-HODE/HETEs produced by tumor cells and endothelial cells appears to modulate the adhesive properties of these cells (Buchanan and Bastida, 1987). Our results support the view that lipoxygenase metabolites of arachidonic acid (12[S]-HETE) and linoleic acid

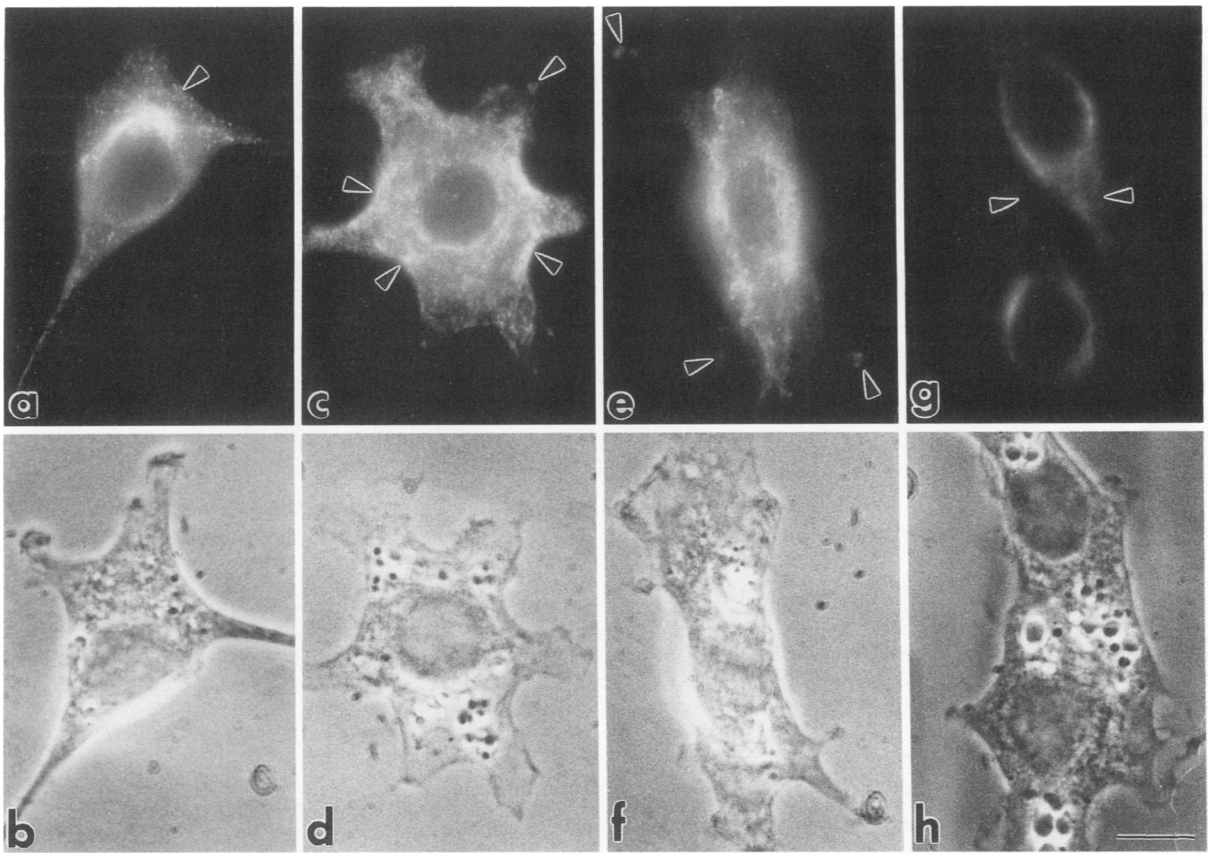

Figure 9. **Immunocytochemical localization of PKC in adherent W256 cells.** PKC was localized with a mAb against rat brain PKC (mAb1.9) as described in Materials and methods. (a) In control W256 cells, PKC is found at the apical plasma membrane as discrete fluorescent foci but not at those membrane areas that are in contact with the substratum (arrows). (c) 12(S)-HETE treatment (250 pmol/10^6 cells, 5 min, 37°C) increased PKC expression (fluorescent areas) at the apical membrane as well as at membrane areas in contact with the substratum (arrows). (e) Effect of 13(R)-HODE pretreatment (250 pmol/10^6 cells, 10 min, 37°C) on 12(S)-HETE-induced PKC translocation in situ. Note the heavy labeling for PKC at the apical membranes as well as in focal contacts (arrows). (g) Effect of 13(S)-HODE pretreatment (250 pmol/10^6 cells, 10 min, 37°C) on 12(S)-HETE induced PKC translocation in situ. Note the weak membrane fluorescence and the lack of immunoreaction at membrane areas in contact with the substratum (arrows). Phase contrast microscopy of cells in (a), (c), (e), and (g) are shown in (b), (d), (f), and (h), respectively. Bars = 10 μm.

(13[S]-HODE) are dual regulators of the adhesive properties of tumor cells to endothelium.

The effect of exogenous 12(S)-HETE on normal cells is well documented (Spector *et al.,* 1988). There are three major cellular activities that are stimulated or altered by 12(S)-HETE: secretion (insulin, renin, corticosteroids, lysosomal enzymes, platelet products), cell motility (in leukocytes, macrophages), and membrane phenotype (expression of C3b-, Fc-, or integrin receptors) (Spector *et al.,* 1988; Grossi *et al.,* 1989). All of these events are regulated in a signal/response coupling manner suggesting the involvement of 12(S)-HETE in signal transduction.

Analyzing the effects of 12(S)-HETE and 13(S)-HODE on W256 cells in this study, we found that only these lipoxygenase metabolites are able to induce or inhibit PKC translocation, respectively. These data are in line with previous reports (Grossi *et al.,* 1989; Schwartz *et al.,* 1990) suggesting that the effect of the PKC activator, PMA, on cell–cell and cell–matrix adhesion may involve lipoxygenase metabolites. Nevertheless, the mechanism for the regulation of PKC by lipoxygenase metabolites is not clear. One possibility is that 12(S)-HETE and/or 13(S)-HODE may be incorporated into membrane phospholipids that influence the activity and localization of PKC. Earlier reports have shown that exogenous 12(S)-HETE is incorporated into the microsomal fraction of cells (Wang *et al.,* 1990) predominantly into phosphatidylcholine or into phosphatidylethanolamine (Suss *et al.,*

1990). However, these phospholipids are not the best-known direct activators of PKC. Interestingly, Legrand and coworkers (1991a,b) have recently reported that 12(S)-HETE and 15(S)-HETE are predominantly incorporated into phosphatidylinositol and phosphatidylcholine, respectively. After incorporation 12(S)-HETE- or 15(S)-HETE-containing diacylglycerols are generated. Although the effect (i.e., activation or inhibition) of these modified diacylglycerols on PKC awaits further study, we speculate that 12(S)-HETE may exert its effect after incorporation into the diacylglycerol moiety. A second possibility is that 12(S)-HETE may bind to a 12(S)-HETE receptor such as has been identified in several cell types (Gross *et al.,* 1990; Suss *et al.,* 1990). The fact that this receptor may be coupled to guanine nucleotide binding proteins (Smith, 1989) provides a link to the mechanisms producing second messengers capable of activating PKC. A third possibility is that 12(S)-HETE itself may serve as an activator of PKC because arachidonic acid as well as its lipoxygenase metabolites, including 12(S)-HETE, can activate PKC in vitro (Hansson *et al.,* 1986; Holian *et al.,* 1989; Shearman *et al.,* 1989; Fan *et al.,* 1990), although the concentrations of 12(S)-HETE used for direct activation are much greater than the concentrations that activate and translocate PKC in intact cells (this study). There is no evidence available for an effect of 13(S)-HODE or related compounds on direct PKC activation, although the parent compound (i.e., linoleic acid) has been shown to activate PKC with moderate effectiveness at high micromolar concentrations (Hansson *et al.,* 1986; Wooton and Wrenn, 1988; Fan *et al.,* 1990). The known second messenger responsible for PKC activation is diacylglycerol. As second messengers, 12(S)-HETE/13(S)-HODE may provide yet another mechanism for bidirectional regulation of PKC and subsequent stimulation or inhibition of tumor cell adhesion to endothelium, a critical event in metastasis.

Materials and methods

Materials

All monohydroxy fatty acids except for 13(R)-HODE (a generous gift from Dr. J. R. Falck, University of Texas, Dallas) were obtained from Cayman Chemical Co. (Ann Arbor, MI) and kept in absolute ethanol at −20°C until use. All protein kinase inhibitors were from Biomol (Plymouth Meeting, PA) except calphostin C, which was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). PMA was from LC Service Corp. (Woburn, MA). [^{14}C]arachidonic acid (54.2 mCi/mmol), [^{14}C]linoleic acid (50 mCi/mmol), and [^{3}H]12(S)-HETE (178 Ci/mmol) were from New England Nuclear (Boston, MA). Mab against rat brain PKC (mAb1.9) was from GIBCO-BRL (Gaitesburg, MD). Streptavidin-Texas Red and Moviol/Citifluor mixture were from Amersham (Buckinghamshire, UK).

Cell lines and tumor cell treatment

W256 cells, a generous gift from Dr. William Orr (McMaster University, Hamilton, Ontario), were maintained as previously described (Chopra *et al.,* 1988). Subconfluent cells (85%) were used in all experiments.

RAECs were isolated, cloned, characterized, and maintained as previously described (Diglio *et al.,* 1982; Menter *et al.,* 1987).

Treatment of W256 cells was performed in Eagle's minimal essential medium (MEM) at 37°C. Control cells were treated with an equivalent amount of diluent (ethanol).

Assay of tumor cell adhesion to endothelium

Adhesion of tumor cells to endothelium was evaluated in a homologous in vitro system (Menter *et al.,* 1987; Grossi *et al.,* 1989). W256 cells (5×10^5) were used for each treatment condition. Treated cells were washed (2 times, MEM) and 1.25×10^5 cells/well were plated onto a confluent RAEC monolayer. The tumor cells and endothelium were incubated for 30 min at 37°C after which time the nonadherent tumor cells were removed by aspiration. The wells were gently washed (2 times, MEM) and the adherent cells were fixed with 4% paraformaldehyde in phosphate-buffered saline. The number of adherent tumor cells in a constant unit area (240 μm^2) was determined by visually counting each well with a Nikon diaphot phase-contrast microscope (Nikon, Garden City, NY). All adhesion assays were run in quadruplicate and repeated a minimum of three times with comparable results.

Measurement of PKC translocation

After treatment of cells, all procedures were performed at 4°C. Treated cells (2×10^7) were washed (2 times) with MEM and then (3 times) with buffer A (20 mM tris(hydroxymethyl)aminomethane (Tris) HCl, pH 7.5, 2 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, 0.15 μM pepstatin A) and finally homogenized in 5 ml of buffer A using a Dounce homogenizer. The 100 000 $\times$ *g* (60 min) supernatants and the Nonidet P-40 solubilized (1%, 30 min) membrane proteins were applied to 1 ml DEAE-cellulose (Sigma, St. Louis, MO) columns equilibrated with buffer B (20 mM Tris HCl, pH 7.5, 2 mM EDTA, 1 mM mercaptoethanol, 0.15 μM pepstatin A). PKC was eluted with 2 ml of buffer B containing 120 mM NaCl. PKC activities in the partially purified cytosolic and membrane fractions were measured using histone III-S (Sigma) as substrate (Gopalakrishina and Barsky, 1988). Briefly, the standard reaction mixture consisted of 20 mM Tris-HCl, pH 7.5, 0.75 mM $CaCl_2$, 10 mM $MgCl_2$, 100 μM [γ^{32}P]ATP (30 Ci/mmol, New England Nuclear, Boston, MA), 25 μg histone III-S, 50 μg/ml leupeptin and PKC preparation, with or without 24 μg phosphatidylserine (Sigma), and 1.6 μg 1.2-dioleoyl-sn-glycerol (Sigma). The reaction was carried out at 30°C for 3 min. PKC activity was calculated by substracting the amount of ^{32}P incorporation into histone III-S observed in the presence of phospholipids and Ca^{2+} from that observed in the absence of phospholipids and in the presence of 5 mM EGTA. Membrane-associated PKC ac-

tivity was expressed as percentage of total activity (cytosol+membrane).

Down-regulation of PKC

Subconfluent W256 cells grown in 150-cm^2 flasks were used. Flasks (3 per treatment) were washed (5 times) with MEM before addition of 50 ml of fresh MEM containing 0.1 μM PMA (final concentration) previously dissolved in absolute ethanol (final concentration of ethanol in MEM 0.002%). An identical amount of ethanol was added to control flasks (3). Cells were incubated with PMA or diluent ethanol at 37°C for 24 h. Afterwards the flasks were washed (5 times, MEM) and cells were removed by a brief treatment with EDTA (7 mM). Both PMA and ethanol-treated cells were washed further (3 times, MEM) and 5×10^5 cells/flask were subjected to treatment with 12(S)-HETE (200 pmol/10^6 cells, 3 min, 37°C) to assess the effect of long-term PMA treatment on their responsiveness to 12(S)-HETE-stimulated adhesion to RAEC. Adhesion assays were performed as described above. PMA or ethanol-treated cells were assayed as described above for total PKC activities (cytosolic+membrane) to determine the degree of down-regulation.

Analysis of lipoxygenase metabolites of arachidonic and linoleic acids in W256 cells

W256 cells (10^7) were incubated in MEM at 37°C with 9 μM [^{14}C]arachidonic acid or 10 μM [^{14}C]linoleic acid for 2, 5, 10, 15, or 30 min. At the end of incubation, 1 N HCl was added to lower the pH of the cell suspension to 3.5 and cells and media were immediately separated by centrifugation (500 $\times$ *g*, 8 min). Metabolites in the media were extracted (2 times) with three volumes of ethyl acetate (Marnett *et al.*, 1991). Cell lipids were extracted by the method of Bligh and Dyer (1959). Extracts were dried under a stream of nitrogen and reconstituted in the appropriate solvent. RP-HPLC was performed using a Beckman Ultrasphere ODS column (Beckman, Fullerton, CA) (4.6 $\times$ 250 mm, 5 μm) with a Vista 5500 pump system (Varian, Palo Alto, CA). Lipoxygenase metabolites of arachidonic acid were resolved by an isocratic solvent system of acetonitrile/water/acetic acid (54:46:0.05) at 1.5 ml/min (Powell, 1985). A similar isocratic solvent system (acetonitrile/water/acetic acid [55:45:0.05]) was run at a flow rate of 1 ml/min to resolve metabolites of linoleic acid (Schade *et al.*, 1987). Column effluent was continuously monitored with a Varian 2550 UV/Vis spectrophotometer (Varian) set at 236 nm and a radioisotope flow detector (β-RAM, IN/US, Fairfield, NJ). Lipoxygenase metabolites of both fatty acids were identified based on retention time of authentic compounds. In the case of 12-HETE, samples were spiked with authentic [^{3}H]12(S)-HETE to confirm the identity of the peak.

Immunocytochemical localization of PKC

PKC in W256 cells was detected with a mAb against rat brain PKC (mAb1.9) that recognizes all three isoforms (Mochly-Rosen *et al.*, 1987). Before using mAb1.9 for immunocytochemical staining, slot blotting was performed to verify the cross-reactivity of this antibody with PKC in cytosolic and membrane preparations of W256 cells prepared as described above. Rat brain cytosol (100 000 $\times$ *g* supernatant) was used as a positive control. Protein concentrations of these preparations were determined by Bradford protein assay (Bradford, 1976) using bovine serum albumin as standard. The blotted nitrocellulose membrane (0.45 μm, Bio-Rad, Richmond, CA) was sequentially incubated with 3% goat whole serum (30 min), mAb1.9 (1 μg/ml, 60 min), 1% goat serum (30 min), biotinylated anti-mouse IgG (5 μg/ml, 30 min). Visualization was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instruction. Negative controls included omission of sample protein, omission of first antibody, and incubation with an inappropriate mouse mAb against the HLADR antigen (AMAC Inc., Westbrook, ME).

For staining PKC in adherent cells, W256 cells were cultured on coverslips for 24 h before immunolabeling. Nonadherent cells were removed with MEM and adherent cells were fixed (30 min) in 4% paraformaldehyde/Hank's balanced saline solution (HBSS) and then permeabilized (2 min) with 0.5% Triton X-100/HBSS followed by 75% ethanol (5 min). After washing (3 times, 5 min) with HBSS, cells were incubated (30 min) with normal goat serum (1:2 in HBSS), mAb1.9 (5 μg/ml in HBSS/4% bovine serum albumin, 60 min, 37°C) and biotinylated goat anti-mouse IgG (1:20 in HBSS/4% bovine serum albumin, 60 min). The complex was visualized by using Streptavidin-Texas Red (1:100 in HBSS/bovine serum albumin). Negative controls included incubation without mAb1.9 or with an inappropriate mouse mAb (MOPC21, Sigma). Coverslips were mounted using a Moviol/Citifluor mixture. Photomicrography was performed with a Leitz Orthoplan microscope using Kodak TMAX400 panchromatic film (Kodak, Rochester, NY).

Statistics

Adhesion and PKC translocation data were analyzed for normality by a one-tailed analysis of variance. When statistically significant differences ($p < 0.05$) were indicated, the results were further analyzed by the Kruskal-Wallis test. Groups with $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using a Macintosh Plus computer (Apple computer, Cupertino, CA) and the STATVIEW 512$^+$ software (Brain Power, Calabasas, CA).

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