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RAPID COMMUNICATIONS

LIPOXYGENASE PRODUCTS REGULATE IRGpIIb/IIIa RECEPTOR MEDIATED ADHESION OF TUMOR CELLS TO ENDOTHELIAL CELLS, SUBENDOTHELIAL MATRIX AND FIBRONECTIN

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Abstract. Tumor cell adhesion to endothelial cells, subendothelial matrix, and fibronectin is stimulated by the lipoxygenase metabolite of arachidonic acid, 12(S)-HETE, but not by 12(R)-HETE, 5-HETE or 15-HETE. Adhesion is also stimulated by the phorbol ester TPA, an effect inhibited by lipoxygenase but not cyclooxygenase inhibitors. TPA and 12(S)-HETE mediated adhesion is due, in part, to an integrin receptor (i.e., IRGpIIb/IIIa) related to the platelet glycoprotein IIb/IIIa complex and is inhibited by specific monoclonal and polyclonal antibodies against platelet IIb/IIIa. TPA and 12(S)-HETE stimulated adhesion is also inhibited by a lipoxygenase product of linoleic acid; i.e., 13-HODE. These results suggest bidirectional control of tumor cell adhesion by lipoxygenase products of arachidonic acid (increase) and linoleic acid (decrease).

Introduction. Metastasis is a complex series of events involving multiple cell-cell (i.e., tumor cell-platelet, tumor cell-endothelial cell, etc.) and cell-matrix (i.e, tumor cell-subendothelial matrix) interactions (1). These interactions are mediated by receptors on the surface of tumor cells, normal cells, and by adhesion proteins which comprise the subendothelial matrix (1). We recently identified a glycoprotein receptor (i.e., IRGpIIb/IIIa) on the surface of human (2), rat (3), and murine (4) tumor cells, including the Lewis lung carcinoma (i.e., 3LL). This receptor is identical to, or immunologically and functionally related to the platelet glycoprotein (Gp) IIb/IIIa complex (2-6). Platelet GpIIb/IIIa is a member of a family of adhesive protein receptors (i.e., integrins; 7) and serves as a multi-functional receptor which interacts with several adhesion proteins [i.e.,fibrinogen Fb, fibronectin FN, and von Willebrand factor (vWF)] (7).

The amount of surface expression of the IRGpIIb/IIIa receptor correlates with metastatic ability, tumor cell ability to induce platelet aggregation, and tumor cell ability to adhere to subendothelial matrix and its components (4,5). We recently reported that a phorbol ester [i.e., phorbol-12-myristate-13-acetate (TPA)] and a lipoxygenase (LOX) metabolite of arachidonic acid [i.e., 12(S,R)-hydroxyeicosatetraenoic acid (12(S,R)-HETE)] increase the surface expression of IRGpIIb/IIIa on human cervical carcinoma cells (5). TPA also increases tumor cell adhesion (5). In the present study, we investigate the effects of TPA and 12-HETE on tumor cell adhesion to endothelial cells (EC), subendothelial matrix (SEM) and a component (i.e., FN) of the SEM.

In addition, we tested for inhibition of adhesion by specific polyclonal and monoclonal antibodies against IRGpIIb/IIIa, 12-LOX inhibitors, and a LOX metabolite of linoleic acid [i.e., 13(S)-hydroxyoctadecadienoic acid; (13-HODE)].

Material and Methods. *Tumor cells and Endothelial cells:* The Lewis lung carcinoma

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was propagated (subcutaneously) in vivo in male C57BL/6J mice, enzymatically dispersed and the tumor cells separated from host cells by centrifugal elutriation as previously described (8). All experiments in this study, employed cells obtained in the elutriated 180 fraction (8). Aortic endothelial cells were isolated from Sprague Dawley rats (Harlan Sprague Dawley Indianapolis, IN) cloned and characterized as previously described (9).

Antibodies, Adhesion Substrates, Eicosanoids and Inhibitors: Polyclonal antibody (pAb) against human platelet IIb/IIIa was elicited in rabbits as previously described (10), and the IgG fraction of pAbIIb/IIIa prepared (9). Monoclonal antibodies (mAb) 10E5 and 7E3 were a generous gift from Dr. B. S. Coller (Stoneybrook , NY). The specificity of these antibodies for IIb/IIIa in rodent cells has been described (2-5,9). Rabbit non-immunized IgG and the IgG fraction of rabbit anti-human transferrin antibody (Cooper Biomedical, Malverin, PA) were used as negative controls in adhesion studies. Goat whole serum (34-37 ng/ml total protein: Cooper Biomedical) was used as a blocking serum for Fc receptors in immunofluorescense studies, and goat anti-rabbit IgG-FITC was used as a secondary antibody. Endothelial cells were allowed to grow to confluence in 24 well plates, removed, and their SEM characterized (9). Fibronectin was a generous gift of Dr. Leo Furcht (University of Minnesota). Lipoxygenase products; 12(S,R)-HETE, 12(R)-HETE, 12(S)-HETE, 5-HETE, and 15-HETE were purchased from Cayman Chemical (Ann Arbor, MI). 13-HODE was synthesized and generously supplied by Drs. Lawrence Marnett and Maddipati Krishnarao (Wayne State University, Detroit, MI). BW755c, NDGA, and indomethacin were obtained from Burroughs Wellcome Research (Triangle Park, NC), Sigma (St. Louis, MO), and the UpJohn Co. (Kalamazoo, MI) respectively.

Adhesion Assays: Adhesion of 3LL cells to EC, SEM, and FN were performend as previouslv described (2, 9, 11) Following elutriation, 3LL cells (180 fraction) were stimulated with TPA (0.1-1.0μM) or lipoxygenase products (0.01-1.0μM) for 15 min prior to addition to wells containing adhesion substrata. In some experiments, cells were stimulated with TPA or 12-HETE in the presence of pAbIIb/IIIa (76μg total protein), mAb10E5 (40μg total protein), 13-HODE (0.1-1.0μM), BW755c (100-250μM), NDGA (100-250μM), or indomethacin (100-250μM) for 15 min prior to their addition to adhesion wells. Cells were incubated (90 min) at 37°C after which time the non-adherent cells were removed by aspiration and the wells washed (3X; PBS). As a control in the antibody experiments, anti-transferrin IgG (an antibody against an unrelated antigen) or IgG from non-immunized rabbit sera was substituted for pAbIIb/IIIa or mAb10E5. To control for the specificity of 12-HETE, cells were stimulated with a product of either the 5-lipoxygenase (5-HETE) or the 15-lipoxygenase (15-HETE). The adherent cells in a constant unit area (unit area = $240\mu m^2$) were visually counted on a Nikon Diaphot inverted phase contrast microscope. All groups were run in triplicate and a minimum of three times with comparable results.

Statistics: Adhesion data were analyzed by the Scheffe's test and by the Kruskal-Wallis Test. Groups with $P<0.05$ were considered statistically significant. All statistical analysis were conducted using a MacIntosh Plus computer (Apple computer, Cupertino, CA) and the STATVEW 512+ software (Brain Power, Calabasas, CA) .

Results and Discussion. We recently reported immunological evidence for a glycoprotein complex related to the platelet GpIIb/IIIa complex on the surface of human colon carcinoma cells and human cervical carcinoma cells (2), in addition to cells derived from several rodent solid tumors (3-5) including 3LL (Fig. 1a, and ref. 4). In addition, we recently performed Northern blot analysis of mRNA isolated from a variety of human and rodent tumor cells using GpIIb and GpIIIa cDNA probes and demonstrated that these tumor cells (including 3LL) expressed specific transcripts which hybridize to the GpIIb cDNA probe and to the GpIIIa cDNA probe (3,6). Finally, in the platelet, GpIIb/IIIa is a calcium dependent heterodimer complex (12), unlike the vitronectin receptor which is a calcium independent complex (2,3,13). Monoclonal antibodies 10E5 and 7E3 recognize epitopes formed when GpIIb and GpIIIa complex and do not recognize the dissociated, monomeric glycoproteins (14). Therefore, we determined if a functional relationship exists between 3LL IRGpIIb/IIIa and platelet GpIIb/IIIa by pretreating 3LL cells with the calcium chelator (EDTA; 0.25mM) prior to probing with mAb10E5 or mAb7E3. A decrease in labeling was observed consistent with the calcium dependence of the IRGpIIb/IIIa complex (mAb10E5, Fig. 1b; mAb7E3, data not shown). The mAb7E3 crossreacts with the vitronectin receptor (3); however, EDTA pretreatment does not reduce binding of mAb7E3 to cells (i.e., endothelial cells) which possess the vitronectin receptor (2,3). Therefore, the decrease in mAb7E3 binding to 3LL cells pretreated with EDTA supports our hypothesis (2,3) that these

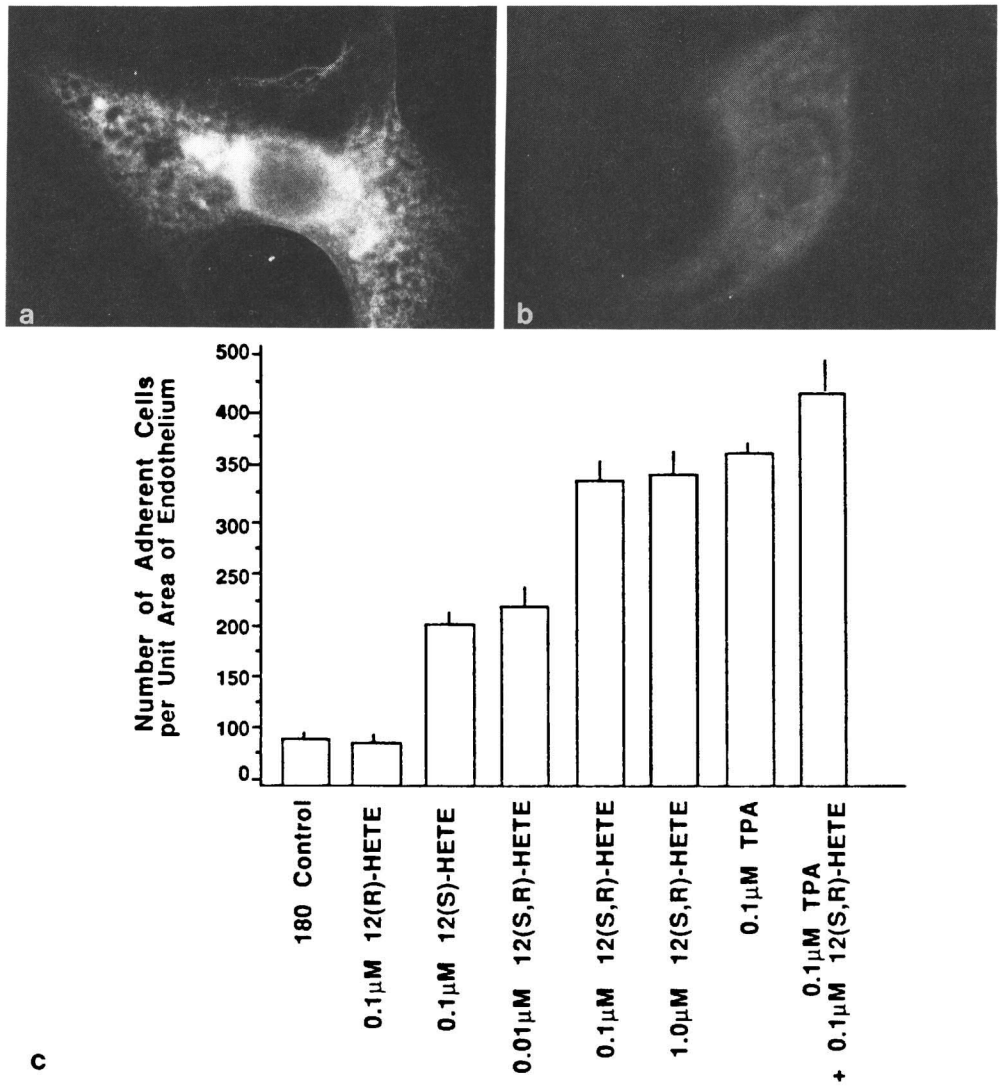

Fig. 1. Immunological localization of IRGpIIb/IIIa on 3LL cells with mAb10E5 (**a**; X250), mAb7E3, and pAbIIb/IIIa (data not shown). Pretreatment of 3LL cells with EDTA decreases binding of mAb10E5 (**b**; X250). (**c**) Effect of stereoisomers of 12-HETE and the effect of TPA on adhesion of 3LL cells to EC monolayers. Bars=mean±S.D.; n=3.

tumor cells, as well as others (2,3), possess a IIb/IIIa like glycoprotein complex, which mediates their adhesion to EC, SEM and its components (i.e., FN). GpIIb and GpIIIa must be complexed to serve as a receptor for adhesion proteins (14). 3LL cells pretreated with EDTA (0.25mM; 15 min) demonstrated decreased adhesion to EC, SEM, and FN (data not shown). The calcium dependence for mAb10E5 and mAb7E3 labeling and the adhesion of tumor cells to EC, SEM, and FN has also been demonstrated for human and rat tumor cells (2,3).

We previously reported that 12-HETE is the major LOX product of arachidonic acid synthesized by 3LL cells (15, 16) and that 3LL 12-HETE biosynthesis is inhibited by LOX inhibitors (i.e., BW755c, NDGA, quercetin, etc.) but not by cyclooxygenase (COX) inhibitors (i.e., indomethacin) (15, 16). Further, TPA (0.01-1.0μM) stimulates 12-HETE biosynthesis with the optimal effect observed at 0.1μM (data not shown). The addition of 5 X 10^5 3LL cells (from the elutriated 180 fraction) to EC coated wells resulted in 97 ± 9 adherent cells/unit area.

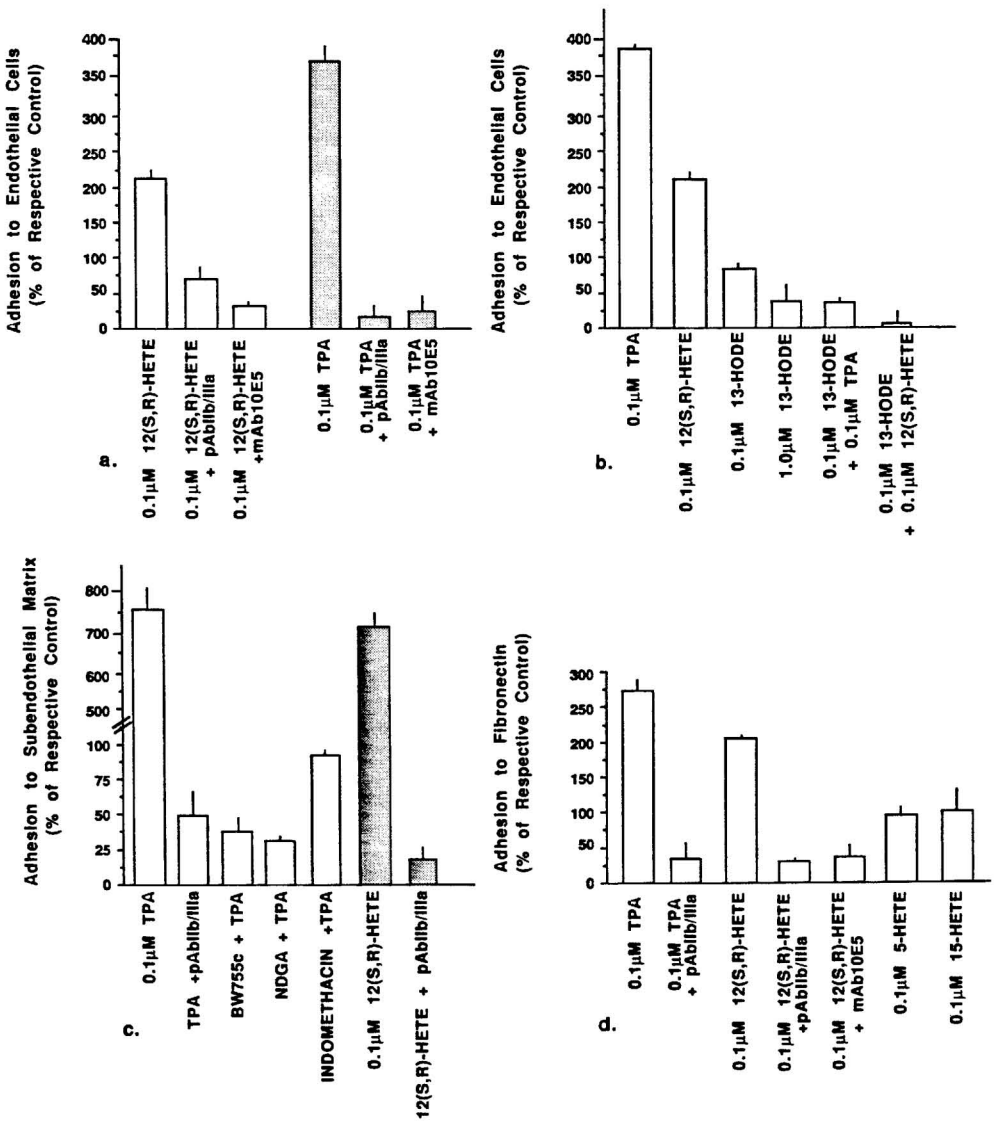

Fig. 2. (a) Inhibition of 12-HETE and TPA stimulated adhesion of 3LL tumor cells to EC by pAbIIb/IIIa or mAb10E5. Bars=mean±S.D.; n=3. Results are expressed as % respective control i.e., 12-HETE and TPA compared to 180 control; inhibitors compared to TPA or 12-HETE stimulated values. (b) 13-HODE inhibition of basal and stimulated 3LL adhesion to EC. Results expressed as in (a). (c) Effects of pAbIIb/IIIa, COX, and LOX inhibitors on TPA and 12-HETE stimulated adhesion to SEM. Results expressed as in (a). (d) Effect of pAbIIb/IIIa and mAb10E5 on TPA or 12-HETE stimulated adhesion to FN. Lack of significant effect of 5-HETE or 15-HETE on adhesion of 180 control. Results expressed as in (a).

The biologically inactive 12-HETE isomer [i.e., 12(R)-HETE] did not increase 3LL adhesion whereas 12(S)- and 12(S,R)-HETE significantly increased adhesion beyond the 180 control, (Fig. 1c). The effect of 12(S,R)-HETE was dose dependent with maximum adhesion observed at 0.1μM (Fig. 1c). TPA (0.1μM) increased 3LL cell adhesion to EC comparable to 12(S,R)-HETE (Fig. 1c). The effect of TPA and 12(S,R)-HETE were not additive (Fig. 1c). Pretreatment of 3LL cells with pAbIIb/IIIa or mAb10E5, prior to their stimulation with 12(S,R)-HETE, reduced adhesion to EC by 66% and 54% respectively (Fig. 2a). PAbIIb/IIIa does crossreact with the vitronectin roceptor, however, mAb10E5 is

specific for IIb/IIIa (2, 3) suggesting that 12(S,R)-HETE enhanced 3LL adhesion to EC is mediated, in part, by the IRGpIIb/IIIa receptor complex. Similarly, TPA enhanced 3LL adhesion to EC was inhibited by pAbIIb/IIIa and mAb10E5 (Fig. 2a). TPA enhanced adhesion of 3LL to EC was reduced by pretreatment of 3LL cells with the LOX inhibitors BW755c and NDGA, but not with the COX inhibitor, indomethacin (data not shown). At present we do not know the identity of the adhesion molecule on the EC surface which interacts with IRGpIIb/IIIa, however EC express vWF (17), an adhesion protein recognized by IRGpIIb/IIIa (data not shown).

Endothelial cells produce a lipoxygenation product of linoleic acid (i.e., 13-HODE) which reduces the adhesion of platelets to their surfaces (18). Therefore we tested exogenous 13-HODE for its ability to reduce basal (180 control) and stimulated [TPA and 12(S,R)-HETE)] adhesion to EC. 13-HODE at 0.1μM and 1.0μM reduced basal 3LL adhesion by 22% and 53% respectively (Fig. 2b). TPA stimulated adhesion was inhibited 71% while 12(S,R)-HETE stimulated adhesion was inhibited 85% by 13-HODE (Fig. 2b).

Tumor cells preferentially adhere to SEM compared to EC (1,4). TPA enhanced 3LL adhesion to SEM 750% above basal adhesion, an effect which was significantly reduced (i.e., 50%) by pAbIIb/IIIa and the LOX inhibitors BW755c (250μM) and NDGA (250μM) but not by the COX inhibitor indomethacin (250μM) (Fig. 2c). Similarly, 12(S,R)-HETE enhanced 3LL adhesion to SEM greater than 12(S,R)-HETE enhanced adhesion to EC; an effect which was significantly reduced (i.e., 80%) by pAbIIb/IIIa (Fig. 2c). TPA and 12(S,R)-HETE enhanced adhesion to SEM was also significantly inhibited by mAb10E5 (data not shown).

Fibronectin is a component of the SEM and an adhesion protein recognized by platelet GpIIb/IIIa (7) and tumor cell IRGpIIb/IIIa (2,4). Both TPA and 12(S,R)-HETE significantly enhanced 3LL adhesion to FN, an effect which was reduced by pAbIIb/IIIa [68% for TPA and 70% for 12(S,R)-HETE)] (Fig. 2d). In order to demonstrate that 3LL adhesion to FN was not due solely to the FN receptor, we pretreated 3LL cells with mAb10E5 prior to 12(S,R)-HETE stimulation. MAb10E5 does not crossreact with the FN receptor (data not shown) and did reduce (i.e., 80%) 12(S,R)-HETE stimulated 3LL adhesion to FN (Fig. 2d). Neither 5-HETE nor 15-HETE significantly affected basal adhesion of 3LL cells to FN, demonstrating the selectivity of the 12-HETE effect (Fig. 2d).

In this study, we present evidence that the 12-lipoxygenase product, 12-HETE, stimulates tumor cell adhesion to EC, SEM, and FN, an effect which is mediated in part by the IRGpIIb/IIIa receptor. This effect is stereochemically specific and not reproduced by products of other lipoxygenases (i.e., 5-LOX or 15-LOX). TPA enhanced adhesion is also mediated in part by the IRGpIIb/IIIa receptor and tumor cell biosynthesis of 12-HETE, as the TPA effect is reduced by LOX but not by COX inhibitors and, 3LL cells synthesize 12-HETE as their major LOX metabolite (15, 16). The results observed with 13-HODE indicated that this LOX metabolite of linoleic acid can antagonize the effects of the LOX metabolite of arachidonic acid (i.e., 12-HETE), suggesting a bidirectional control of tumor cell adhesion possibly at the level of the IRGpIIb/IIIa receptor. We are currently examining the relative amounts of 12-HETE and 13-HODE synthesized by tumor cells possesing different metastatic ability, and found that 12-HETE biosynthesis increases with the increased tumor cell ability to adhere to SEM and FN, and increased tumor cell ability to metastasize (5). These results have significant implications for hematogenous metastasis, as successful tumor cell arrest and adhesion to the vessel wall are considered critical events in the metastatic cascade (1).

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