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Autocrine Motility Factor Signals Integrin-mediated Metastatic Melanoma Cell Adhesion and Invasion[1]

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ABSTRACT

The binding of autocrine motility factor (AMF) to its cell surface receptor, gp78, stimulates tumor cell motility. In this report, we provide evidence that stimulation of gp78 by either AMF or a monoclonal antibody to gp78 (3F3A) increases adhesion and spreading of metastatic murine melanoma (B16a) cells on fibronectin. This gp78-regulated increase is mediated by up-regulation of surface αIIbβ3 and α5β1 integrin receptors. In addition, AMF treatment of B16a cells increased translocation of αIIbβ3 and α5β1 from the cytoplasm to the cell surface. However, αIIbβ3 and α5β1 demonstrate separate and unique staining patterns at the surface of B16a cells in response to stimulation of gp78. Furthermore, stimulation of B16a cells with AMF increased their invasion through Matrigel. This stimulated invasion was inhibited by antibodies to αIIbβ3 but not by antibodies to α5β1. The increased integrin surface expression and function in response to AMF was blocked by *N*-benzyl-*N*-hydroxy-5-phenylpentanamide, an inhibitor of 12-lipoxygenase, and calphostin C, an inhibitor of protein kinase C. The results demonstrate that AMF stimulates integrin-mediated B16a cell adhesion, spreading, and invasion, and these events are regulated by a signaling pathway involving 12-lipoxygenase and protein kinase C.

INTRODUCTION

Metastasis is a complex, yet well co-ordinated, process that can be defined by a series of integrated events (1): (*a*) tumor cells must adhere and spread on the extracellular matrix; (*b*) proteinases are necessary to degrade the matrix; and (*c*) tumor cells must migrate through the degraded matrix. Adhesion and spreading of tumor cells on the matrix is primarily mediated by integrins (1–4). These receptors also play a pivotal role in initiating the proteolytic degradation of the matrix (5), thereby facilitating tumor cell invasion.

The β1 and β3 subfamilies of integrins play an important role in tumor invasion and dissemination (6). Qualitative and quantitative changes in expression of the fibronectin receptor (α5β1) are involved in the proliferative response of quiescent human melanoma cells (5, 7), whereas overexpression of β3 integrins directly correlates with metastatic potential in melanoma (2). The integrin αIIbβ3 initially was believed to be expressed only on platelets (3). However, by using reverse transcription-PCR, Northern and Southern blotting, and immunoprecipitation, we demonstrated that this integrin receptor is expressed on metastatic B16a melanoma cells (8). Furthermore, it was demonstrated that this receptor plays an important role in tumor cell-platelet, tumor cell-endothelial cell, and tumor cell-ECM[4] interactions (9–13). The integrin αIIbβ3 is expressed in human colon adenocarcinoma (14), prostate, melanoma, and breast cancer cell lines,[5] suggesting that αIIbβ3 may play an important role in tumor progression and metastasis.

Given the importance of integrins in various steps of the metastatic cascade, it is logical that factors that regulate integrin expression and function may have a significant impact on the metastatic process. Integrin expression is controlled at the transcriptional level as well as the posttranscriptional levels. Several transcriptional regulators of integrin gene expression have been reported in the literature. Regulators such as transforming growth factor β (15), interleukin 1 (16), IFN-γ (17), tumor necrosis factor-α (17), basic fibroblast growth factor (18), and platelet-derived growth factor (19) positively or negatively regulate integrin gene expression. However, these regulators require treatment of several hours to days to alter integrin gene expression. A more rapid form of integrin regulation is at the posttranscriptional level. This change in integrin expression is qualitative because it involves receptor translocation from an intracellular pool to the plasma membrane and *vice versa*. Mechanisms involved in posttranscriptional regulation of integrins are not as well characterized as the transcriptional regulators. Previous data demonstrate that treatment of tumor cells with the 12-LOX metabolite of arachidonic acid, 12(*S*)-HETE, induces posttranscriptional surface expression of integrins within 15 min (9, 10, 20–22). This effect is achieved primarily by increased translocation of the integrin from the cytoplasm to the cell surface (22). In addition, 12(*S*)-HETE is also capable of activating transcription of integrin genes as reported for αv gene expression in microvascular endothelial cells (23).

Cumulative reports demonstrate that 12(*S*)-HETE is involved in all of the three major aspects of tumor cell invasion, *i.e.*, adhesion, release of matrix degradative proteinases, and motility (2, 9, 10, 22). Exogenous 12(*S*)-HETE stimulates tumor cell motility (24), whereas endogenously generated 12(*S*)-HETE mediates motility in response to AMF (25). AMF is a member of a family of tumor cell cytokines that stimulates motility (26) via a receptor-mediated signaling pathway (27–30). Signal transduction following AMF binding to its cell surface receptor, gp78 (AMFR), is mediated by a pertussis toxin-sensitive G protein (31), phosphorylation of gp78 itself (28), inositolphosphate production (32), and activation of 12-LOX-mediated signaling, which involves activation of PKC (24, 25). Since AMF activates the 12-LOX signaling pathway, we questioned whether it could also regulate integrin expression and function. In this report, we provide evidence that AMF not only regulates motility, but it also modulates integrin-mediated tumor cell adhesion, spreading, and invasion.

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[4] The abbreviations used are: ECM, extracellular matrix; AMF, autocrine motility factor; COX, cyclooxygenase; 12-LOX, 12-lipoxygenase; PFA, paraformaldehyde; mAb, monoclonal antibody; PKA, cyclic AMP-dependent kinase; PKC, protein kinase C; 12(*S*)-HETE, 12-(*S*)-hydroxyeicosatetraenoic acid; BHPP, *N*-benzyl-*N*-hydroxy-5-phenylpentanamide; SFM, serum-free media; ASA, acetylsalicylic acid.

[5] Trikha, M., Chen, Y. Q., Timar, J., Szekeres, C., Bazaz, R., and Honn, K. V., unpublished observations.

MATERIALS AND METHODS

Murine Melanoma Cell Culture. Murine B16a melanoma cells were obtained from the Animal and Human Tumor Bank National Cancer Institute (Frederick, MD). Tumor cells were cultured in RPMI containing 5% fetal bovine serum (Life Technologies, Inc., Bethesda, MD) and antibiotics (22). Confluent cells were harvested with 0.45 M EDTA and HBSS (EDTA/HBSS).

AMF Purification. AMF was purified from HT1080 human fibrosarcoma conditioned media as described previously (24). Briefly, semiconfluent HT1080 cells were washed with PBS and cultured in SFM for 24 h. This medium was replaced with fresh SFM, which was collected after 24 h. Conditioned medium was pooled, centrifuged, concentrated, and purified by molecular sieve chromatography as described (24).

Antibodies. Rat monoclonal IgM antibody (3F3A), which stimulates AMF receptor (gp78) signaling, was prepared as described previously (27). Adhesion blocking mAb to the fibronectin receptor (anti-α5) was purchased from Oncogene Science (Uniondale, NY). A polyclonal antibody to the fibronectin receptor $\alpha5\beta1$ and mAb to the $\beta1$ integrin subunit were purchased from Life Technologies, Inc. Nonimmune rat serum or IgG (MOPC 21) was purchased from Sigma Chemical Co. (St. Louis, MO). AP-4 (mAb to αIIb; Ref. 8), OPG-2 (mAb to $\beta3$; Ref. 8), and AP-2 (mAb to $\alpha IIb\beta3$ complex; Refs. 8 and 33) were generous gifts from Dr. Thomas Kunicki (The Scripps Research Institute, La Jolla, CA).

Tumor Cell Adhesion and Spreading Assay. B16a cells in exponential growth phase were harvested with EDTA/HBSS and washed extensively with SFM. After washing, tumor cells were incubated at 37°C for 1 h prior to plating on fibronectin (5 μg/well)-coated 24-well plates. Fibronectin coating of 24-well plates was performed by air drying wells containing 5 μg of protein in PBS. Following incubation, B16a cells (10^5 cells/500 μl SFM) were incubated on the fibronectin-coated well for 60 min at 37°C. After incubation, wells were washed three times with PBS to remove nonadherent cells. The remaining adherent cells were fixed with 1% PFA/PBS for 10 min. Two approaches were used to quantitate the number of adherent cells: (*a*) adherent cells per unit area were counted using a phase contrast microscope (×200); (*b*) adherent cells were stained with Giemsa stain for 5 min, after which excess stain was washed off, and absorbance at 600 nm was recorded by a Bio-Rad plate reader (Model 3550; Bio-Rad, Richmond, CA).

Cell spreading was determined as described previously (10). Briefly, adherent cells were examined under ×400 magnification, and the cell population was divided into round, intermediate, and fully spread groups. The spreading factor was calculated by obtaining a ratio of the number of spread to completely round cells. Three randomly selected areas per well were counted. For every data point, at least 100 cells were counted, and each data point was performed in triplicate.

Flow Cytometry of B16a Cells. B16a cells were detached with EDTA/HBSS, washed three times with SFM, and allowed to recover for 1 h in a tissue culture incubator. B16a cells (1×10^6) were treated with 3F3A (20 μg/ml) or nonimmune rat serum (1:25 dilution) for 15 min at 37°C and subsequently washed with SFM and fixed with 1% PFA/HBSS for 10 min. After fixation, cells were blocked with normal goat serum (1:2 dilution) for 30 min and then incubated with AP-2 (20 μg/ml) or mAb1 (20 μg/ml) for 1 h. Bound primary antibody was detected with goat antimouse IgG conjugated to biotin (1:200 dilution; Amersham, Arlington Heights, IL) and streptavidin-FITC (1:200 dilution; Amersham). Fluorescence of labeled B16a cells (1×10^4) was measured in a Coulter Epic flow cytometer (Coulter, Hialeah, FL), and the mean fluorescence was determined. Background fluorescence of labeled B16a cells was determined by incubating tumor cells with either nonimmune rat serum (1:25 dilution) or secondary antibody in the absence of primary antibody.

Immunofluorescence. B16a cells were plated on a fibronectin-coated glass surface for 60 min in the presence or absence of AMF (1.25 ng/ml) or 3F3A (20 μg/ml). Intracellular proteins were detected by fixing the cells with a methanol/acetone solution at −20°C (25). Plasma membrane proteins were detected by fixing tumor cells with 1% PFA/PBS solution for 10 min. After fixation, cells were permeabilized with 0.5% Triton X-100 for 3 min at room temperature. Nonspecific binding was blocked by incubating immobilized cells with normal goat serum diluted 1:2 in PBS for 30 min. $\alpha IIb\beta3$ integrin was detected by AP-2 (20 μg/ml), and fibronectin receptor ($\alpha5\beta1$) was detected by mAb 1 (20 μg/ml). Bound primary antibodies were detected with goat antimouse IgG conjugated to biotin (1:100 dilution) and streptavidin-Texas Red. Stained cells were visualized through a Nikon epifluorescence microscope at ×100 magnification and photographed on Kodak Tmax 400 black and white film. Direct labeling of B16a cells with secondary antibody-biotin conjugate and streptavidin-Texas Red exhibited no significant background staining.

B16a Melanoma Cell Invasion through Matrigel. This assay was performed in Matrigel-coated Biocoat polycarbonate filter inserts (4 μm; Collaborative Research, Bedford, MA). These inserts were placed on top of 500 μl of AMF containing SFM in a 24-well plate. B16a cells (5×10^4/200 μl) in SFM were added to the upper chamber of the insert. Tumor cells were incubated in a tissue culture incubator for 12 h. Following incubation, tumor cells in the upper chamber were scraped off, and the bottom of the polycarbonate filter was stained with Diffquick solution (American Scientific Products, McGraw Park, IL). The filter was removed from the insert and mounted on glass coverslides. Tumor cells that migrated through the insert were counted under a light microscope. Each data point was performed in triplicate.

Drug Treatment of B16a Cells. H8 (Seigaku America, Rockville, MD) was used to inhibit PKA activity at a concentration of 50 μM (10). Calphostin C (Calbiochem, La Jolla, CA) at a concentration of 5 μM was used to inhibit PKC activity (24). ASA (300 μM; Ref. 10) and BHPP (1 μM; Refs. 10 and 34) were used to inhibit COX and 12-LOX activity, respectively. Optimal inhibitor concentrations were determined in previous experiments reported in the above-cited references.

RESULTS

Effect of AMF on B16a Cell Adhesion and Spreading on Immobilized Fibronectin. B16a cells (2×10^5/ml) after treatment with various concentrations of AMF were added to fibronectin-coated wells, and the cell adhesion assay was performed as described in "Materials and Methods." The number of adherent cells per unit area was counted through a phase contrast microscope. AMF in a dose-dependent manner stimulated adhesion of B16a cells to immobilized fibronectin (Fig. 1*A*). Maximum AMF-stimulated adhesion to fibronectin was observed at a concentration range of 0.5–2.5 ng/ml.

Since AMF stimulated B16a cell adhesion to fibronectin, we questioned whether it also could influence cell spreading. AMF in a dose-dependent manner increased spreading of B16a cells on fibronectin (Fig. 1*B*). At doses of 0.25–2.5 ng/ml, AMF induced greater than an 8-fold increase in cell spreading as compared to spreading in the absence of AMF.

$\alpha IIb\beta3$ and $\alpha5\beta1$ Are Involved in AMF-stimulated B16a Cell Adhesion to Immobilized Fibronectin. To determine which integrins are involved in AMF-stimulated B16a cell adhesion to fibronectin, we used adhesion blocking mAbs to the $\beta1$ and $\beta3$ subfamilies of integrins. Both mAbs to the $\beta1$ and to the $\beta3$ integrins inhibited basal cell adhesion to fibronectin (in the absence of gp78 stimulation) as well as gp78-stimulated cell adhesion (Fig. 2). These findings suggest that AMF-stimulated B16a cell adhesion to fibronectin may involve both the $\beta1$ and $\beta3$ subfamily of integrins. To further determine which integrin receptor within the $\beta3$ subfamily is involved in AMF-stimulated cell adhesion, we used adhesion blocking antibodies to $\alpha IIb\beta3$. mAbs to αIIb (AP-4; Fig. 2) and $\alpha IIb\beta3$ (AP-2; data not shown) block AMF-stimulated cell adhesion to fibronectin. Furthermore, antibodies to the fibronectin receptor (polyclonal antibody to $\alpha5\beta1$; data not shown) and mAb 1 (anti-$\alpha5$) also inhibit AMF-stimulated B16a cell adhesion to fibronectin (Fig. 2). Adhesion blocking antibody to $\alpha v\beta3$ was not used because we have determined previously that these cells lack αv message (8). These results, collectively, suggest that B16a cell adhesion to fibronectin in the presence or absence of AMF involves at least the $\alpha5\beta1$ and $\alpha IIb\beta3$ integrin receptors.

AMF Treatment of B16a Cells Increases Surface Expression of $\alpha IIb\beta3$ and $\alpha5\beta1$. Flow cytometry was used to determine whether the AMF-induced increase in adhesion and spreading was mediated by an up-regulation of $\alpha5\beta1$ and/or $\alpha IIb\beta3$. Tumor cells were treated

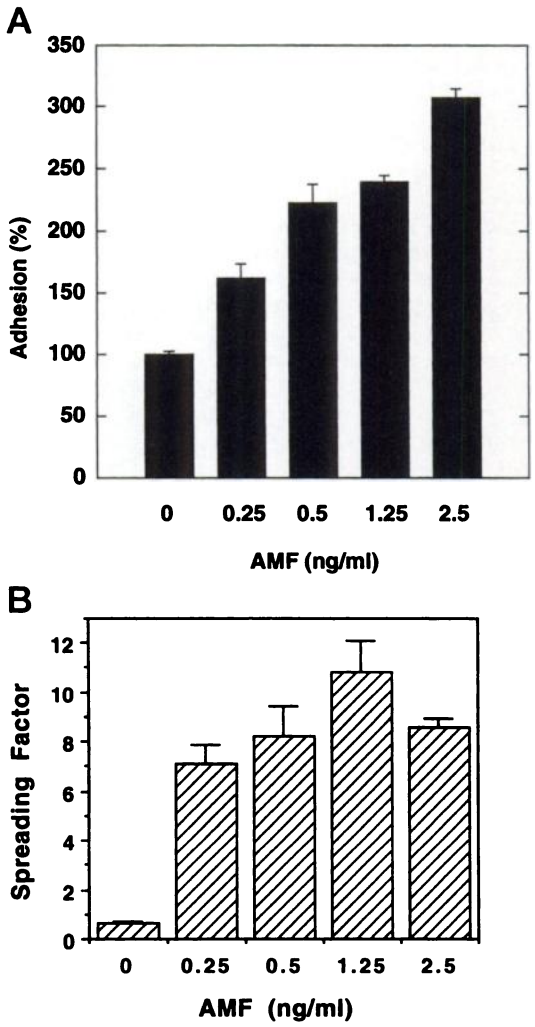

Fig. 1. Dose-dependent stimulation by AMF of B16a cell adhesion and spreading on fibronectin. B16a cells (1×10^5/500 μl) were suspended in SFM or varying concentrations of AMF and added in triplicate to 24-well plates precoated with 5 μg of fibronectin. *A*, dose-dependent stimulation of adhesion of B16a cells to fibronectin. A cell adhesion assay was performed as described in "Materials and Methods." Results are expressed as the percentage increase in adhesion in the presence of various concentrations of AMF with respect to adhesion in the absence of AMF. *B*, dose-dependent stimulation by AMF of B16a cell spreading on fibronectin. B16a cells were treated with varying concentrations of AMF as in *A*, and a cell spreading assay was performed as described in "Materials and Methods." Results are expressed as the increase in spreading factor, which was determined as a ratio of spread to round cells. Three random areas were counted for adhesion and spreading, and each data point is expressed as the mean of triplicate determinations; *bars*, SD.

with normal rat serum (1:25 dilution) or 3F3A (an AMF receptor-stimulating antibody) for 15 min and fixed with 1% PFA/PBS. Surface expression of αIIbβ3 and $\alpha5\beta1$ was detected by AP-2 and mAb 1, respectively. 3F3A treatment of B16a cells increased surface expression of both integrin receptors (Fig. 3). These findings suggest that AMF-increased adhesion and spreading of B16a cells on fibronectin may correlate with increased surface expression of αIIbβ3 and $\alpha5\beta1$.

Activation of gp78 Differentially Regulates Cell Surface Localization of $\alpha5\beta1$ and αIIbβ3. Flow cytometric analysis indicated that stimulation of gp78 receptor results in increased surface expression of $\alpha5\beta1$ and αIIbβ3. Therefore, we used immunofluorescent staining to investigate whether this increase in surface expression of $\alpha5\beta1$ and αIIbβ3 was due to receptor translocation from the cytoplasm to the cell surface. To visualize the intracellular $\alpha5\beta1$ integrin pool, B16a cells were fixed with methanol/acetone and labeled with mAb 1 in the presence or absence of 3F3A (Figs. 4, *a* and *b*). 3F3A treatment dramatically altered the cytoplasmic localization of $\alpha5\beta1$. In the absence of 3F3A treatment, B16a cells demonstrated a distinct perinuclear staining pattern for the fibronectin receptor (Fig. 4*a*). However, after the addition of 3F3A, the fibronectin receptor stained with a diffuse cytoplasmic pattern (Fig. 4*b*). A similar response was observed for an intracellular αIIbβ3 pool when adherent B16a cells were labeled with AP-2 (data not shown). These morphological observations were quantitated by evaluating the ratio of dispersed *versus* perinuclear integrin staining in the tumor cell population. Table 1 indicates that treatment of B16a cells with 3F3A increased by 4-fold

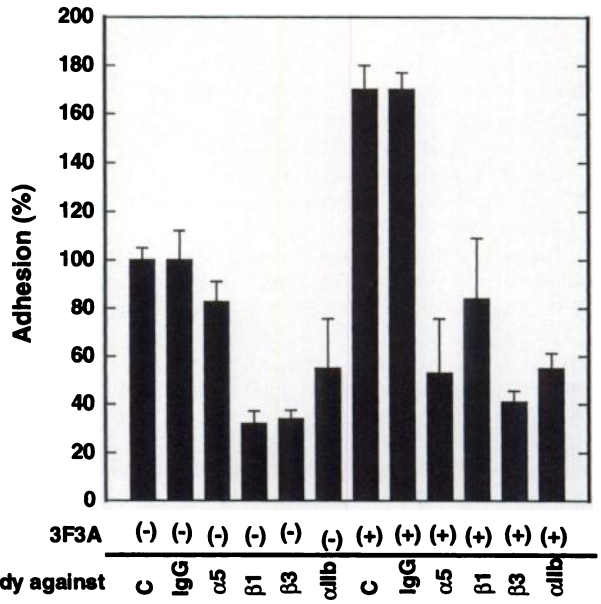

Fig. 2. Inhibition of AMF-stimulated B16a cell adhesion to fibronectin by anti-integrin antibodies. B16a cells were preincubated in the presence or absence of nonimmune antibody, MOPC21 (20 μg/ml), or the following adhesion blocking antibodies: anti-fibronectin receptor antibody (anti-$\alpha5$; mAb 1, 20 μg/ml), anti-$\beta1$ (20 μg/ml), anti-$\beta3$ (OPG-2, 20 μg/ml), or anti-αIIb (AP-4, 20 μg/ml) in the presence or absence of 3F3A (20 μg/ml). A cell adhesion assay was performed as described in "Materials and Methods." Adherent cells were stained with Giemsa, and absorbance at 600 nm was recorded. Results are expressed as the change in the percentage of adhesion in the presence of various antibodies with respect to adhesion in the absence of antibody. Each data point represents the mean of triplicate determinations; *bars*, SD.

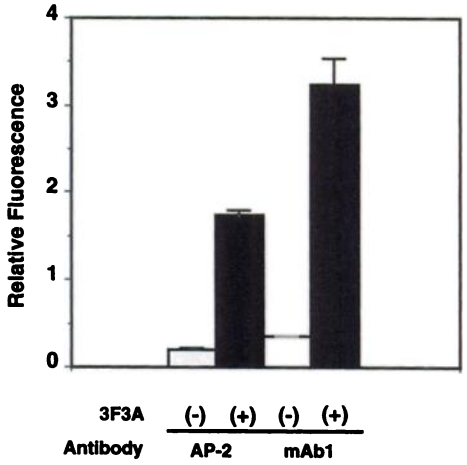

Fig. 3. gp78-stimulated increase in integrin surface expression on B16a cells. B16a cells (1×10^6) were treated with normal rat serum (1:25 dilution) or anti-AMF receptor antibody (3F3A, 20 μg/ml) for 15 min. After incubation, surface expression of αIIbβ3 and $\alpha5\beta1$ was detected by AP-2 and mAb 1, respectively. Flow cytometry was performed as described in "Materials and Methods." Results are expressed as the mean of triplicate determinations of relative fluorescence; *bars*, SD.

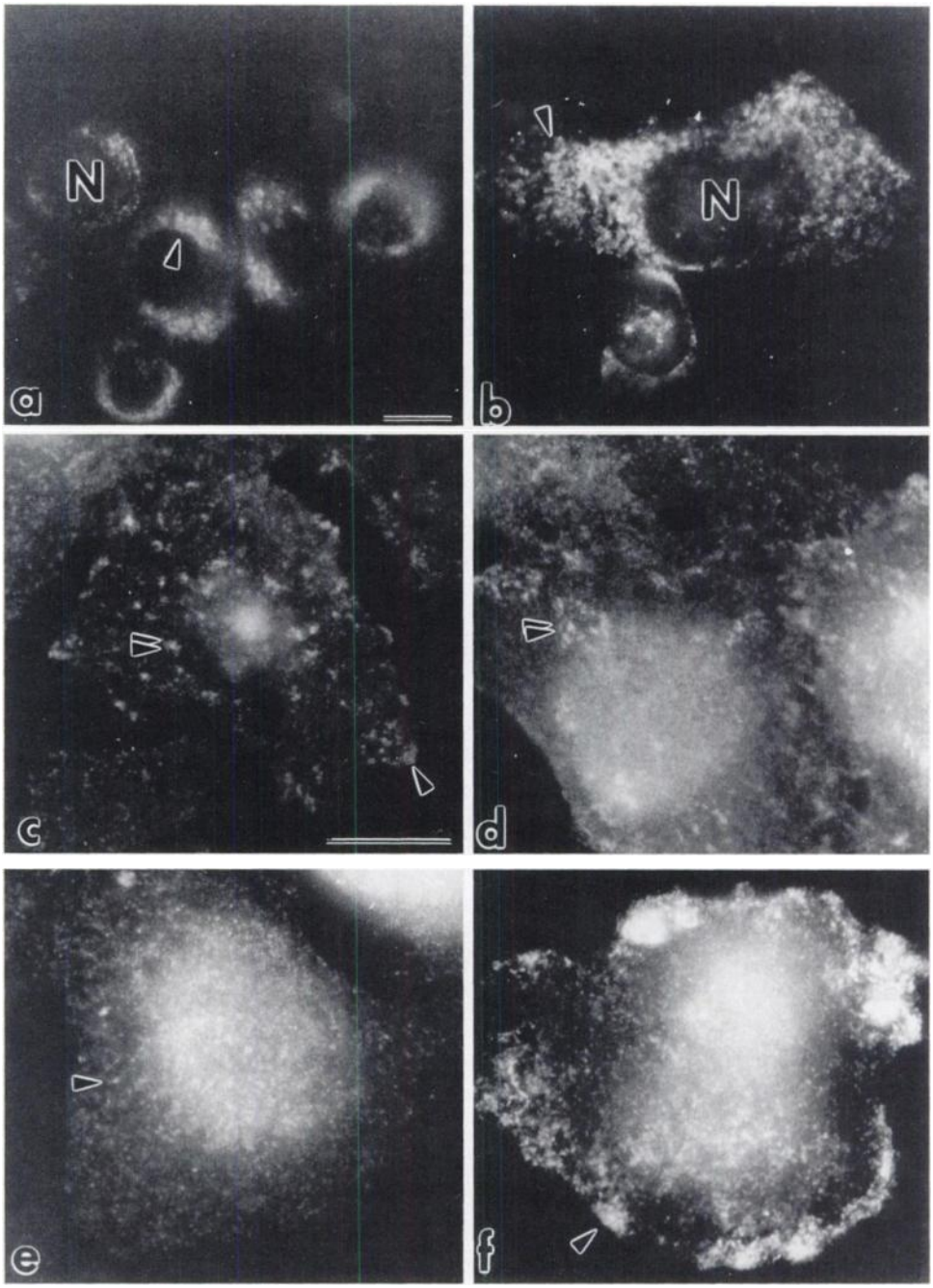

Fig. 4. Effect of gp78 ligation on cellular localization of αIIbβ3 and α5β1 on B16a cells. Tumor cells were incubated on fibronectin-coated coverslips and stimulated with either nonimmune rat serum (1:25 dilution), 3F3A (20 μg/ml), or AMF (1.25 ng/ml) for 60 min at 37°C. Integrins in the cytoplasm or at the plasma membrane were detected by fixing adherent tumor cells with methanol/acetone (*a* and *b*) or PFA/Triton (*c-f*), respectively. Following fixation, fibronectin receptor and αIIbβ3 were detected by mAb 1 and AP-2, respectively. Immunofluorescence was performed as described in "Materials and Methods." Detection of a cytoplasmic pool of α5β1 receptor in untreated cells (*a*) and in 3F3A-treated cells (*b*). Detection of α5β1 receptor at the plasma membrane in untreated cells (*c*) and in AMF-treated cells (*d*). Detection of αIIbβ3 at the plasma membrane in untreated cells (*e*) and in AMF-treated cells (*f*). *Single arrowhead,* integrin localization at the cell periphery; *double arrowhead,* integrin localization at areas resembling adhesion contacts. *N,* nucleus; *bar,* 10 μm.

the number of cells with a dispersed α5β1 pattern and by 7-fold in cells with a dispersed αIIbβ3 pattern, suggesting that stimulation of gp78 induces translocation of integrins to the cell surface. Another interpretation of this finding is that AMF treatment induces a change in cell shape (Fig. 1*B*) that alters staining of integrin receptors without inducing translocation of integrins from an intracellular pool to the cell surface. However, flow cytometry (Fig. 3) indicates that 3F3A treatment of tumor cells increased surface expression of α5β1 and αIIbβ3, and in addition, it has been reported that membrane translocation of integrins can occur during cell spreading (10, 22). These findings, collectively, suggest that stimulation of gp78 in B16a cells results in increased cell spreading and translocation of integrins from the perinuclear zone to the cell periphery.

Since stimulation of gp78 resulted in an increased surface expression of α5β1 and αIIbβ3, we determined subcellular staining for these integrins. Fibronectin-adherent B16a cells were permeabilized with Triton X-100 and labeled with antibodies to the fibronectin receptor (mAb 1) or to αIIbβ3 (AP-2). B16a cells in the presence or absence of AMF displayed no significant change in subcellular localization of α5β1 (Figs. 4, *c* and *d*). In contrast, labeling of αIIbβ3 in untreated B16a cells was dramatically different when compared to AMF-treated cells. In the absence of AMF stimulation, staining of αIIbβ3 appeared to be uniformly distributed along the apical cell membrane (Fig. 4*e*). In contrast, staining for αIIbβ3 in the AMF-treated cells was concentrated to the peripheral regions of the cell membrane (Fig. 4*f*). These results demonstrate that AMF differentially regulates the localization of α5β1 and αIIbβ3.

αIIbβ3 Is Involved in AMF-stimulated B16a Invasion through Matrigel. Since antibodies to αIIbβ3 and to α5β1 inhibited gp78-stimulated tumor cell adhesion (Fig. 2), we characterized their effect on tumor cell invasion through Matrigel. AMF (1.25 ng/ml) stimulated B16a cell invasion through Matrigel, and antibodies to αIIb, β3,

Table 1 *Morphometric analysis of intracellular distribution of αIIbβ3 and α5β1 in B16a cells*[a]

Integrin	Dispersion ratio[b]	
	Control	3F3A-treated
α5β1	0.20 ± 0.02	0.93 ± 0.35
αIIbβ3	0.30 ± 0.09	2.09 ± 0.40

[a] B16a cells that had adhered to fibronectin were stimulated with 3F3A (20 μg/ml) or nonimmune rat serum (1:25 dilution) for 60 min at 37°C, fixed with methanol/acetone, and stained for the cytoplasmic pool of α5β1 or αIIbβ3 as described in "Materials and Methods."

[b] Dispersion ratio, the intracellular distribution of αIIbβ3 and α5β1, was evaluated in 100 cells, and a dispersion ratio was determined. The dispersion ratio was calculated by obtaining a ratio of the number of cells demonstrating a dispersed intracellular integrin staining pattern: the number of cells demonstrating a perinuclear integrin staining pattern. Data are the means of triplicate determinations ± SD.

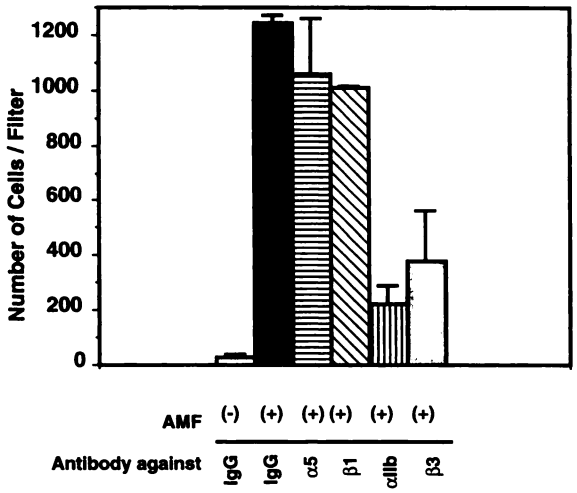

Fig. 5. Inhibition of AMF-stimulated invasion by anti-integrin antibodies. B16a cells (5×10^4) were added to Matrigel-coated filters in the absence or presence of AMF (1.25 ng/ml) and the following antibodies: nonimmune IgG (MOPC21, 20 μg/ml), anti-fibronectin receptor (anti-α5; mAb 1, 20 μg/ml), anti-β1 (20 μg/ml), anti-αIIb (AP-4, 20 μg/ml), or anti-β3 (OPG-2, 20 μg/ml). Tumor cell invasion assay was performed as described in "Materials and Methods." Results are expressed as the number of cells that migrated through the Matrigel-coated filter. Each data point is the mean of triplicate determinations; *bars*, SD.

and αIIbβ3 inhibited this invasion (Fig. 5). However, adhesion-blocking antibodies to the fibronectin receptor (mAb 1) or anti-β1 did not block AMF-stimulated tumor cell invasion (Fig. 5). The number of B16a cells that invaded through Matrigel in the absence of AMF was so small that the effect of anti-integrin antibodies on basal invasion could not be determined accurately. These findings suggest that αIIbβ3, but not α5β1, may be involved in AMF-stimulated tumor cell invasion through Matrigel.

Role of Arachidonic Acid Metabolites in gp78 Signaling. Previous studies demonstrated that AMF stimulates endogenous 12-LOX protein expression and 12(*S*)-HETE production (25), suggesting that 12-LOX inhibitors may suppress gp78-mediated signaling. Therefore, events believed to be downstream of gp78-mediated signaling were classified into the following three categories: integrin expression; adhesion; and spreading. To investigate the role of arachidonic acid metabolism in these events, BHPP (1 μM) and ASA (300 μM) were used as 12-LOX and COX inhibitors, respectively. Pretreatment of B16a cells (15 min) with BHPP inhibited gp78-mediated increase in αIIbβ3 surface expression by approximately 50% (Fig. 6). In contrast, ASA (300 μM) treatment of B16a cells did not inhibit the gp78-mediated increase in surface expression of αIIbβ3 (Fig. 6). In addition, BHPP (1 μM), but not ASA (300 μM), blocked gp78-stimulated B16a cell adhesion and spreading to immobilized fibronectin (Table 2). Collectively, the data suggest that gp78 signaling is coupled to 12-LOX activity but not to COX activity.

Effect of PKA and PKC Inhibitors on AMF-stimulated B16a Cell Adhesion and Spreading. It has been reported that AMF-induced tumor cell motility involves PKC, but not PKA, stimulation (24, 25); therefore, we questioned whether AMF-stimulated cell adhesion was mediated via the PKC or the PKA signaling pathway. To address this question, H8 was used to inhibit PKA activity, and calphostin C was used to inhibit PKC activity. Incubation of B16a cells with calphostin C (5 μM) resulted in a complete inhibition of 3F3A-induced adhesion of tumor cells to fibronectin, whereas treatment of tumor cells with H8 (50 μM) partially blocked 3F3A-stimulated B16a cell adhesion to fibronectin (Table 3). In addition, calphostin C, but not H8, blocked the spreading of B16a cells on fibronectin (Table 3). These findings, in combination with previous reports (24, 25), suggest that gp78-dependent adhesion, spreading, and motility are primarily coupled to PKC.

DISCUSSION

Integrins play a critical role in tumor cell growth and metastasis as a result of their involvement in tumor cell adhesion, spreading, and

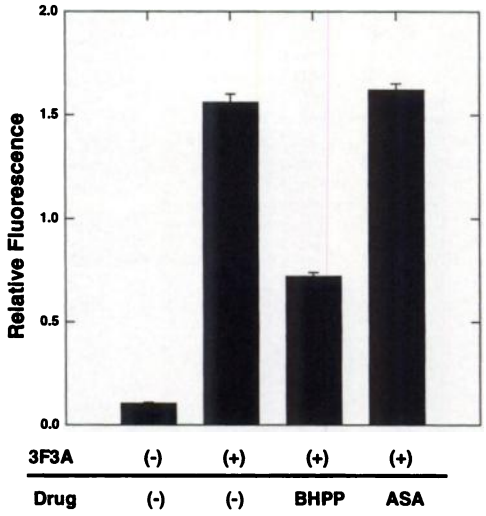

Fig. 6. Inhibition of gp78-induced increase in αIIbβ3 integrin expression on B16a cells by 12-LOX and COX inhibitors. B16a cells (1×10^6) were incubated with 3F3A (20 μg/ml) in the presence of BHPP (1 μM) or ASA (300 μM) for 15 min. Following incubation, flow cytometry was performed on tumor cells as described in "Materials and Methods." Surface expression of αIIbβ3 was detected by AP-2 (20 μg/ml). Results are expressed as the mean of triplicate determinations of relative mean fluorescence; *bars*, SD.

Table 2 *Role of 12-LOX and COX inhibitors in 3F3A-stimulated tumor cell adhesion and spreading*

B16a cells, in the presence of nonimmune rat serum (1:25 dilution, control) or 3F3A (20 μg/ml), were treated with BHPP (1 μM), ASA (300 μM), or diluent (ethanol), and the cells were allowed to adhere to fibronectin. The cell adhesion and spreading assay was performed as described in "Materials and Methods." The results are expressed as the means of triplicate determinations ± SD.

Drug	Control		3F3A	
	Adhesion (%)[a]	Spreading factor[b]	Adhesion (%)	Spreading factor
Ethanol	100 ± 9	1.23 ± 0.31	190 ± 12	5.26 ± 0.41
ASA	ND[c]	ND	154 ± 15	4.81 ± 0.13
BHPP	ND	ND	82 ± 17	2.26 ± 0.18

[a] Adhesion was obtained by calculating the percentage of change in cell adhesion in the presence of drug with respect to adhesion in the presence of the control diluent (ethanol).

[b] Calculated by obtaining a ratio of the number of spread to round cells that were adhered to fibronectin.

[c] ND, not determined.

Table 3 *Role of protein kinase inhibitors in 3F3A-stimulated B16a cell adhesion and spreading*

B16a cells, in the presence of nonimmune rat serum (1:25 dilution, control) or 3F3A (20 μg/ml), were treated with H8 (50 μM), calphostin C (5 μM), or diluent (ethanol), and the cells were allowed to adhere to fibronectin. The cell adhesion and spreading assay was performed as described in "Materials and Methods." The results are expressed as the means of triplicate determinations ± SD.

	Control		3F3A	
Drug	Adhesion (%)[a]	Spreading factor[b]	Adhesion (%)	Spreading factor
Ethanol	100 ± 9	0.25 ± 0.06	191 ± 14	5.21 ± 1.29
H8	ND[c]	ND	132 ± 10	6.05 ± 1.09
Calphostin C	ND	ND	60 ± 15	0.33 ± 0.09

[a] Adhesion was obtained by calculating the percentage of change in cell adhesion in the presence of drug with respect to adhesion in the presence of the control diluent (ethanol).

[b] Calculated by obtaining a ratio of the number of spread to round cells that were adhered to fibronectin.

[c] ND, not determined.

migration (1, 2, 7, 35). Therefore, the discovery of signal transduction pathways that regulate integrin expression and function may have a major impact on tumor progression and invasion. The 12-LOX metabolite of arachidonic acid, 12(*S*)-HETE, enhances integrin-mediated tumor cell adhesion to endothelial cells (2, 35) and to the ECM (2, 10, 35). Previously, we demonstrated that B16a cells possess the integrin αIIbβ3, and they use this receptor to adhere to endothelial cells and to the subendothelial matrix (9–13). This adhesion could be enhanced with 12(*S*)-HETE treatment due to the increased translocation of αIIbβ3 from a cytoplasmic pool to the cell surface (9, 22).

The literature indicates that AMF-stimulated tumor cell motility also can be induced by 12(*S*)-HETE, and this response is coupled to 12-LOX and PKC signaling events (24, 25). Since this signaling pathway is involved in integrin expression and function (9, 10, 20–22), we hypothesized that AMF should also enhance integrin expression and function on tumor cells. In this study, we report for the first time that AMF mimics the 12(*S*)-HETE-mediated response in tumor cells in terms of stimulating tumor cell adhesion, spreading, invasion, and up-regulation of surface integrins. Results from flow cytometry suggest that the AMF receptor (gp78)-mediated increase in tumor cell adhesion and spreading is a consequence of surface up-regulation of αIIbβ3 and α5β1. Furthermore, pretreatment of B16a cells with antibodies to α5β1 and αIIbβ3 abrogate AMF-stimulated tumor cell adhesion, thereby demonstrating that AMF-stimulated tumor cell adhesion and spreading involve α5β1 and αIIbβ3. This is in accordance with our previous study reporting that 12(*S*)-HETE-stimulated B16a cell adhesion and spreading also use αIIbβ3 (10), implying that AMF and 12(*S*)-HETE participate in the same intracellular signaling pathway.

Results from flow cytometry and immunofluorescence indicate that stimulation of gp78 can induce translocation of both α5β1 and αIIbβ3 receptors from the cytoplasm to the cell surface. Interestingly, it appears that stimulation of gp78 by either AMF or 3F3A, similar to the addition of exogenous 12(*S*)-HETE (10), differentially regulates subcellular localization of α5β1 and αIIbβ3. Immunofluorescent staining indicates that AMF treatment of B16a cells results in no significant change in subcellular localization of α5β1. In contrast, membrane labeling of αIIbβ3 after AMF treatment is markedly different from the staining of αIIbβ3 in untreated cells. In the absence of AMF, αIIbβ3 is uniformly distributed at the apical cell surface, whereas after AMF treatment, αIIbβ3 distinctly localizes to the peripheral region of the cell membrane. Why α5β1 and αIIbβ3 localize differently at the cell surface in response to AMF is presently unclear. We speculate that other signaling pathways that do not involve gp78, 12-LOX, and/or PKC may regulate surface distribution of α5β1.

Both AMF and 3F3A stimulate the motility of several metastatic tumor cells, including B16a cells (24, 25, 27–29). In this study, we demonstrate for the first time that AMF stimulates invasion of B16a cells through Matrigel and that antibodies to αIIbβ3, but not to α5β1, inhibit this AMF-induced invasion. Seftor *et al.* (36) reported that invasion of human melanoma cells through Matrigel involves αvβ3 because antibodies to this receptor stimulated invasion. B16a cells do not express message for αv but instead express the αIIbβ3 integrin receptor (8). In contrast with the findings of Seftor *et al.* (36), we observed that antibodies to αIIbβ3 inhibited AMF-stimulated invasion of B16a cells through Matrigel. Currently, the role of αvβ3 in AMF-stimulated melanoma cell invasion is under investigation in our

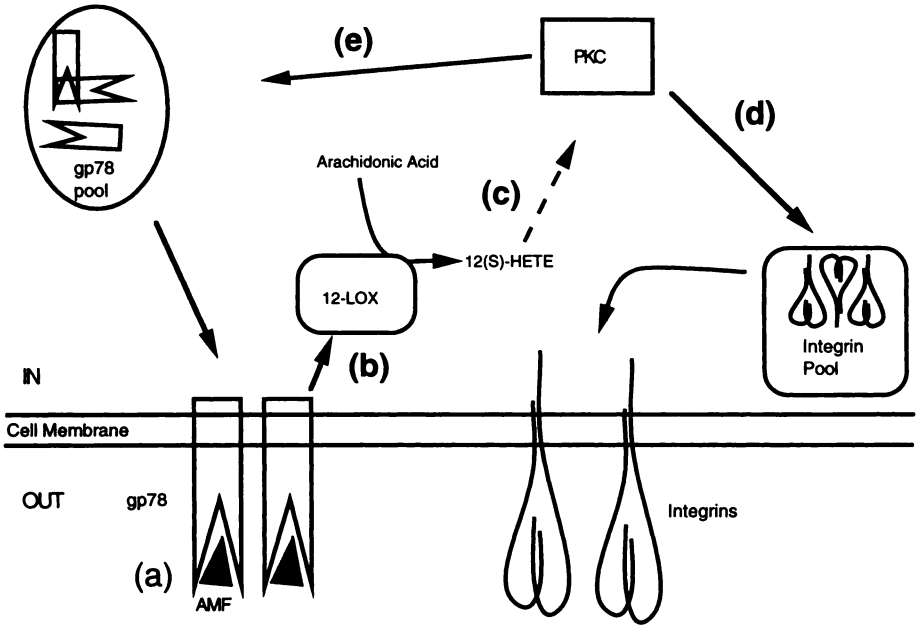

Fig. 7. Schematic representation of the proposed "autocrine motility signaling pathway" that results in integrin-mediated tumor cell adhesion, spreading, and invasion. Signal transduction begins when AMF binds to its receptor gp78 *(a)*, thereby activating 12-LOX *(b)*, which results in the production of 12(*S*)-HETE, and it activates PKC *(c)*. Activation of PKC results in translocation of the cytoplasmic pool of integrins *(d)* and gp78 *(e)* to the cell surface. Increased integrin expression at the cell surface regulates adhesion, spreading, and invasion. *Dotted lines* indicate that these molecules are not directly connected to each other.

laboratory. Nonetheless, these observations imply that $\alpha v\beta 3$ and $\alpha IIb\beta 3$ may transmit different intracellular signals during the process of tumor cell invasion. The finding that antibodies to $\alpha 5\beta 1$ did not block AMF-stimulated invasion could be attributed to at least two possibilities: (*a*) the concentration of fibronectin in Matrigel may be too insignificant to support binding of $\alpha 5\beta 1$ on B16a cells; and (*b*) as mentioned above, surface localization of $\alpha 5\beta 1$ may not be coupled to the gp78 signaling cascade.

gp78-stimulated tumor cell adhesion, spreading, and invasion are dependent on 12-LOX activity. This is supported by the fact that a selective 12-LOX inhibitor BHPP (10, 34) blocked gp78-mediated increase in surface expression of $\alpha IIb\beta 3$. Also, BHPP inhibited adhesion and spreading of B16a cells on fibronectin. Furthermore, BHPP inhibited AMF-stimulated B16a invasion through Matrigel (data not shown). In contrast, a COX inhibitor, ASA, had no effect on AMF-stimulated surface upregulation of $\alpha IIb\beta 3$, adhesion, or spreading. These results in combination with previous reports (24, 25) demonstrate that 12-LOX is part of the gp78 signaling pathway, which is involved in AMF-stimulated tumor cell adhesion, spreading, and invasion.

Our previous reports demonstrated that 12-LOX is upstream of PKC because 12(*S*)-HETE activates PKC (10, 21, 24, 34). Since gp78-mediated signaling activates 12-LOX (24, 25), we predicted that ligation of this receptor should also activate PKC. A PKC inhibitor, calphostin C, blocks gp78-stimulated tumor cell adhesion and spreading. Furthermore, AMF treatment of B16a cells induces a translocation of PKC from the cytoplasm to the peripheral membranes (data not shown). These findings suggest that gp78-mediated signaling is coupled to 12-LOX, which in turn activates PKC in B16a cells. Based on data from this study in combination with previous reports, we propose the existence of an "autocrine motility signaling pathway" involving (Fig. 7): (*a*) AMF binding to its receptor gp78; (*b*) signaling of 12-LOX to synthesize 12(*S*)-HETE (Fig. 6; Table 2; Refs. 24 and 25); (*c*) 12(*S*)-HETE-induced activation of PKC (10, 21, 24, 34); (*d*) translocation of PKC from subcellular structures to lipid membranes (34); (*e*) PKC-dependent translocation of $\alpha IIb\beta 3$, $\alpha 5\beta 1$, and gp78 from intracellular pools to the cell surface (Fig. 4; Tables 1 and 3; Refs. 22 and 25); and (*f*) differential surface localization of $\alpha 5\beta 1$ and $\alpha IIb\beta 3$ (Fig. 4) and increased surface expression of gp78 (24), which contributes to enhanced tumor cell adhesion, spreading, and invasion.

Tumor cell invasion is a complex multistep process that requires regulated expression of cellular adhesion molecules such as integrins. Our data demonstrate that in B16a cells, AMF regulates integrin-dependent tumor cell adhesion, spreading, and invasion by using the 12-LOX-PKC signaling pathway. Detailed analysis of this signaling pathway will further enhance our understanding of tumor cell invasion.

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