

Emodin Inhibits Tumor Cell Adhesion through Disruption of the Membrane Lipid Raft-Associated Integrin Signaling Pathway

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Abstract

Cell adhesion and spreading is a crucial step in the metastatic cascade of cancer cells, and interruption of this step is considered to be a logical strategy for prevention and treatment of tumor metastasis. Emodin is the major active component of the rhizome of *Rheum palmatum* L., with known anticancer activities. Here, we first found that emodin significantly inhibited cell adhesion of various human cancer cells. This inhibition was achieved through suppressing the recruitment of focal adhesion kinase (FAK) to integrin β_1 as well as the phosphorylation of FAK followed by the decreased formation of focal adhesion complex (FAC). In understanding the underlying mechanisms, we found that emodin inhibited the lipid raft clustering and subsequent colocalization of integrin β_1 and FAC proteins within lipid rafts. Lipid profile analysis revealed significant decrease of cholesterol and sphingolipids in raft fraction after emodin treatment. Cholesterol replenishment abolished the adverse effect of emodin on the translocation of integrin β_1 and FAC proteins into the lipid raft fraction and cell adhesion. Therefore, data from this study provide novel evidence that emodin inhibits cell adhesion and spreading through disruption of the membrane lipid raft-associated integrin signaling pathway. (Cancer Res 2006; 66(11): 5807-15)

Introduction

Cancer metastasis consists of a complex cascade of biological events, including adhesion, migration, and invasion, which finally allow tumor cells to escape from primary site and invade and proliferate at ectopic environments (1, 2). Cell adhesion and spreading is a crucial step in the metastatic cascade of cancer cells. Interruption of this step is considered to be a logical strategy for prevention and treatment of tumor metastasis.

It has been well established that cell adhesion and spreading is mediated by a variety of transmembrane proteins, including integrins, cadherins, selectins, and intercellular adhesion molecules (1, 2). Among these adhesion molecules, the integrins and their downstream signaling pathways have been extensively studied. Integrins are α/β heterodimeric membrane proteins that facilitate the anchorage of cells to the components of extracellular matrix (ECM; ref. 3). The attachment of cells to certain ECM proteins, such as fibronectin, collagen, and laminin, leads to the clustering of integrins with subsequent formation of focal adhesion (4). Several

cytoplasmic proteins are recruited into focal adhesions, such as focal adhesion kinase (FAK), c-Src, paxillin, and vinculin (5). Specifically, FAK is a key regulator of cell adhesion and migration. Interaction of β subunit of integrins and FAK causes kinase autophosphorylation at Y397 of FAK where c-Src targets. c-Src further fully phosphorylates and activates FAK, which recruits additional structural and signaling molecules to contribute to the assembly of focal adhesion complex (FAC; refs. 5, 6).

Lipid rafts are distinct plasma membrane microdomains, composed of cholesterol tightly packed with sphingolipids, particularly sphingomyelins, and may serve as signaling platforms to recruit essential proteins for intracellular signal transduction and coordinate transmembrane signaling and cell adhesion (7, 8). Importantly, integrins have been recently found to be lipid raft associated (9). The function of integrins, such as integrin $\alpha_4\beta_1$, was reported to be positively regulated by the lipid rafts in T lymphocytes (10). The attachment of cells to ECM proteins is deficient when cells are totally devoid of sphingolipids (11).

Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) is one of the main active components contained in the root and rhizome of *Rheum palmatum* L. It inhibits the activity of HER-2 protein tyrosine kinase (12), p56^{lck} (13), and phosphatidylinositol 3-kinase (14). Recent studies suggest that emodin could also induce apoptosis in several kinds of cancer cells (15, 16). Our previous work showed that emodin exhibited strong inhibitory effect on cancer cell migration (17) and invasion (18). Because cell adhesion is an important event in the metastatic cascade of cancer cells, here, we further examined the effect of emodin on cancer cell adhesion. Our finding shows that emodin strongly inhibits the adhesion of various cancer cells. More importantly, this inhibition is achieved through suppressing lipid raft coalescence and subsequent interference of integrin clustering and FAC formation.

Materials and Methods

Cell lines. MDA-MB-231 (a human breast cancer cell line), HeLa (a human cervix epitheloid carcinoma cell line), and HepG2 (a human hepatocarcinoma cell line) were purchased from American Type Culture Collection (Manassas, VA). HSC5, derived from human skin squamous carcinoma, was kindly provided by Dr. S. Kondo (Yamagata University, Yamagata, Japan). MDA-MB-231, HeLa, and HSC5 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). HepG2 cells were grown and passaged in MEM containing 10% FBS.

Chemicals and reagents. Emodin, laminin, collagen I, fibronectin, soluble cholesterol, methyl- β -cyclodextrin (MBCD), and anti-vinculin antibody were purchased from Sigma (St. Louis, MO). Emodin was dissolved in DMSO before use. Antibodies against paxillin, FAK, phosphorylated FAK (Y397), and c-Src were purchased from Santa Cruz Technology (Santa Cruz, CA). Sepharose 4B was from Amersham Biosciences (Uppsala, Sweden). Purified human integrins $\alpha_1\beta_1$ and $\alpha_5\beta_1$ and antibodies against integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_1\beta_1$, and β_1 were from Chemicon (Temecula, CA). Alexa Flour 488-conjugated cholera toxin subunit B (CTxB) and antibody against transferrin receptor (TfR) were from Invitrogen Life Technologies

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(Carlsbad, CA). Lipid internal standards (cholesterol, ceramide, ceramide glucoside, and sphingomyelin) were purchased from Avanti Polar Lipids (Alabaster, AL), and [26,26,26,27,27-d₆] cholesterol was from CDN Isotopes (Pointe-Claire, Quebec, Canada).

Cell adhesion and spreading assays. Adhesion and spreading assays were done as described previously with modifications (19). Ninety-six-well plates were coated with fibronectin (5 µg/mL), collagen I (5 µg/mL), laminin (10 µg/mL), or heat-denatured bovine serum albumin (BSA; 1%) at 4°C overnight and then blocked in BSA (1%) for 1 hour. Cells were harvested using cell dissociation solution (Sigma) and resuspended in FBS-free medium/0.1% BSA. Cells were then treated according to the context for 1 hour. Control sample was treated with 0.1% DMSO in culture medium, which is equal to DMSO concentration in emodin-treated samples. For the cell adhesion assay, cells (2×10^5 /mL) were seeded onto the plates with different coatings as described above and incubated for 20 minutes at 37°C. Nonadherent cells were removed by gentle washing with PBS. Adherent cells were fixed and stained with 0.1% crystal violet in 20% methanol. Incorporated dye was dissolved in 10% acetic acid, and the absorbance was measured at 560 nm. The results were presented as percentage attachment, where 100% attachment corresponds to the attachment of untreated cells exposed to fibronectin, collagen, or laminin, respectively.

For the cell spreading assay after designated treatment, cells were seeded onto the plates with different coatings. After 1 hour of incubation, cells were fixed without washing. Spread cells were defined as those cells that had lost their phase-bright appearance and had readily distinguishable nucleus and cytoplasm and quantified by counting six randomly selected fields in each well under a phase-contrast microscope.

Immunocytochemical staining and visualization of lipid rafts. Cells were pretreated for 1 hour according to the context and seeded onto coverslips coated with fibronectin. After designated time intervals, cells were fixed with 2% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100, and blocked with 2% BSA for 30 minutes. Incubation with primary antibodies was done overnight at 4°C. Visualization was done using fluorescently tagged secondary antibodies. The labeling of membrane lipid rafts with CTxB was conducted as reported previously with slight modifications (20). Briefly, cells were fixed in 2% paraformaldehyde for 20 minutes on ice followed by incubation with 5 µg/mL Alexa Flour 488-conjugated CTxB for 20 minutes. The costaining of integrin β_1 was then done as described above.

Separation of proteins between detergent-soluble and detergent-insoluble fractions. Separation was conducted as described previously with modifications (21). In brief, after designated treatments, cells were lysed in ice-cold lysis buffer [150 mmol/L NaCl, 0.5% Triton X-100, 20 mmol/L Tris (pH 7.5)] with proteinase inhibitor cocktail (Roche, Nutley, NJ). The lysates were centrifuged at $14,000 \times g$ for 30 minutes at 4°C, and the supernatants were collected as the detergent-soluble fraction. The detergent-insoluble pellets were resuspended and briefly sonicated in the same lysis buffer supplemented with 0.5% SDS and 2 mmol/L DTT.

Coimmunoprecipitation and Western blot. Cell lysates were prepared with coimmunoprecipitation lysis buffer [50 mmol/L HEPES (pH 7.6), 250 mmol/L NaCl, 0.1% NP40, 5 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, proteinase inhibitor cocktail]. Cell lysates (500 µg) were first incubated with 5 µg designated antibodies for 1 hour at 4°C followed by incubation with protein A-Sepharose beads for another 1 hour at 4°C. The beads were washed thrice with ice-cold coimmunoprecipitation lysis buffer, resolved by SDS-PAGE, and immunoblotted with antibodies against various proteins of interest.

Measurement of cell surface integrins with flow cytometry. After designated treatments, cells were fixed with 2% paraformaldehyde in PBS and then incubated with antibodies against integrin $\alpha_1\beta_1$, $\alpha_2\beta_1$, or $\alpha_5\beta_1$ for 1 hour. As a negative control, cells were stained with isotype-matched immunoglobulin G (IgG). Cells were then incubated with FITC-conjugated secondary antibody for another 1 hour and resuspended in 500 µL PBS for flow cytometry analysis.

Solid-phase binding assay with purified integrins. Solid-phase binding assay was done as described previously with minor modifications (22). Briefly, 96-well plates were coated with fibronectin (5 µg/mL), collagen

I (5 µg/mL), laminin (10 µg/mL), or BSA (1%), all dissolved in coating buffer [20 mmol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂] at 4°C overnight. The plates were then blocked with 1% BSA in coating buffer for 1 hour at room temperature. Integrin $\alpha_5\beta_1$ (0.2 µg) or $\alpha_1\beta_1$ (0.2 µg) was then added and incubated with or without emodin for 2 hours at 37°C. After washing away the unbound integrins, primary antibody against integrin β_1 was added to the plates and incubated for 2 hours followed by incubation with secondary antibody [anti-mouse IgG-horse-radish peroxidase (HRP)] for 1 hour. HRP activity was determined with 3,3',5,5'-tetramethylbenzidine/H₂O₂ as a substrate, and absorbance was measured at 450 nm. The amount of nonspecific binding was determined using wells coated with BSA alone.

Lipid extraction, electrospray ionization/mass spectrometry, and atmosphere pressure chemical ionization analysis of lipids. Lipid extraction was done using the Bligh and Dyer method (23). Mass spectrometry (MS) analysis of cholesterol was carried out at atmosphere pressure chemical ionization (APCI)-positive mode with an Applied Biosystems 4000 Q-Trap MS (Applied Biosystems, Foster City, CA). Multiple reaction mechanism (MRM) transitions of 369/161 and 375/161 were established for quantification of cholesterol.⁴ Briefly, 20 µL sample was introduced into the MS using an Agilent high-throughput liquid chromatography (HTLC) system (Agilent Technologies, Palo Alto, CA) with chloroform/methanol (1:1) as a mobile phase at a flow rate of 200 µL/min. The APCI conditions were vaporizer temperature (500°C) and corona discharge current (3 µA). For method validation, different amounts of cholesterol standards were added into cell extracts to test the linearity. The intensities of individual ions were compared with their corresponding internal standard species. The relative content of each individual ion in untreated insoluble fraction was normalized to 1, and the results were expressed as relative content of treated groups compared with that of control group. Based on electrospray ionization (ESI)/MS, ESI/MS/MS, and ESI/MS/MS/MS data obtained from a Q-ToF MS (Waters, Milford, MA) and an Applied Biosystems 400 Q-Trap MS, major sphingolipids in insoluble fractions were analyzed using MRM at ESI-negative mode on the Q-Trap MS. MRM transitions for sphingolipids were set up based on fragmentation patterns of ceramide heads of sphingolipids (24, 25). Typically, 20 µL sample was introduced into the MS using an Agilent HTLC system with chloroform/methanol (1:1) as a mobile phase at a flow rate of 200 µL/min. The ion spray voltage and temperature were set at 4,500 V and 250°C, respectively. Nitrogen was used as curtain gas (value of 20), and collision gas was set to high.

Statistical analysis. The results obtained from each experiment are expressed as mean \pm SD of triplicates. The significance level was set at $P < 0.05$ for each analysis using Student's *t* test.

Results

Effect of emodin on cancer cell adhesion and spreading. In our previous studies, emodin has been found to possess strong inhibitory effect on the migration (17) and invasion (18) of cancer cells. As cell adhesion is one of the essential steps involved in cancer metastasis, here, we further investigated the effect of emodin on cancer cell adhesion. We first examined the effect of emodin on cell adhesion to fibronectin, a major ECM component (4). The experiment was conducted using four different cancer cell lines, including MDA-MB-231, HSC5, HepG2, and HeLa cells. As shown in Fig. 1A, the presence of fibronectin on the culture surface significantly enhanced cell adhesion compared with BSA. Emodin suppressed cancer cell adhesion to fibronectin dose dependently. We also measured the effect of emodin on cell adhesion to collagen I and laminin, two other major components of ECM (4). Emodin showed similar inhibitory effect in a dose-dependent fashion in

⁴ G. Shui et al., in preparation.

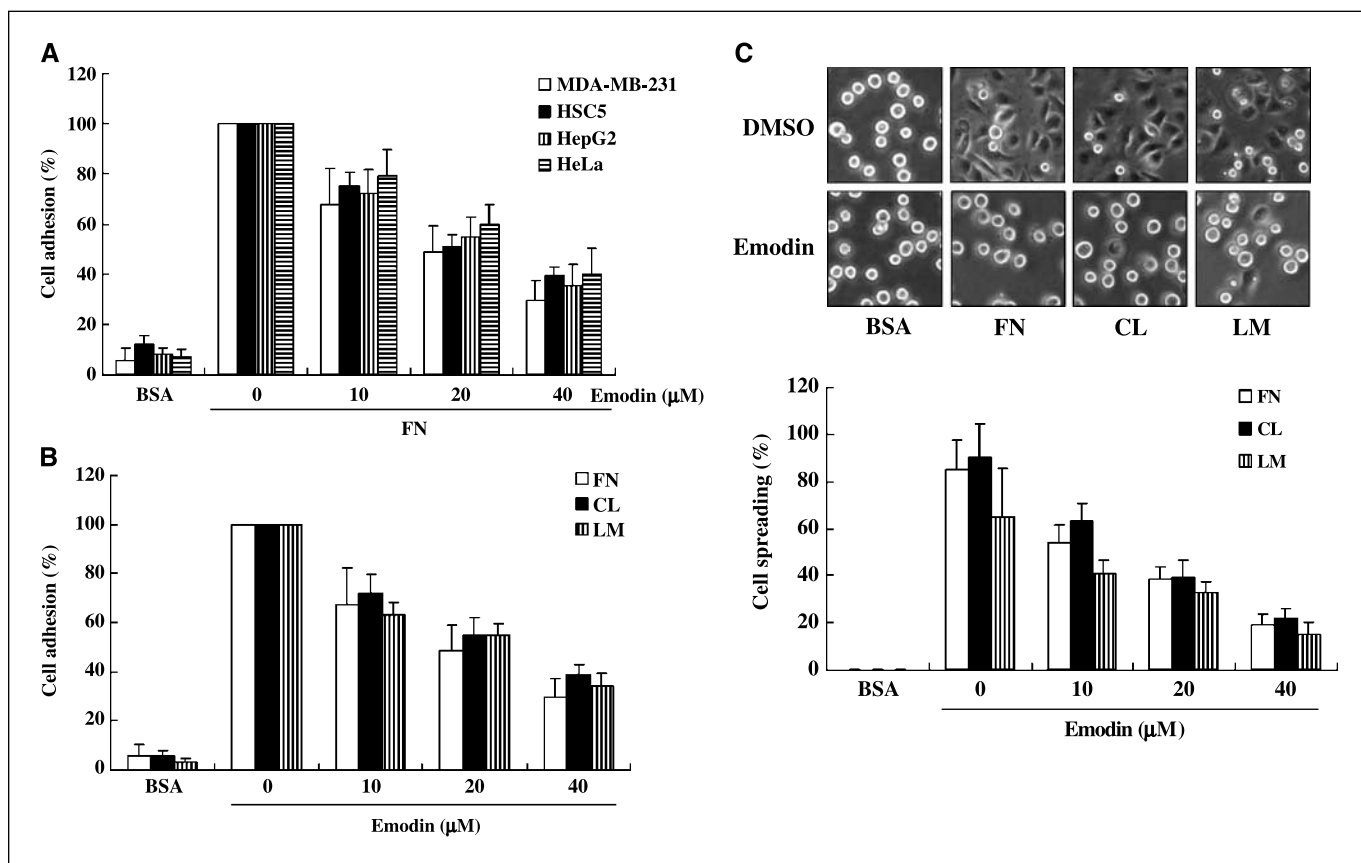


Figure 1. Emodin inhibits cancer cell adhesion and spreading. Serum-starved human cancer cells were harvested and pretreated with emodin (0–40 $\mu\text{mol/L} \times 1$ hour) and then seeded onto 5 $\mu\text{g/mL}$ fibronectin (FN), 5 $\mu\text{g/mL}$ collagen I (CL), 10 $\mu\text{g/mL}$ laminin (LM), or 1% BSA-coated 96-well plates. Adhesion and spreading assays were conducted as described in Materials and Methods. **A**, inhibitory effect of emodin on adhesion of MDA-MB-231, HSC5, HepG2, and HeLa cells to fibronectin. **B**, suppression of emodin on adhesion of MDA-MB-231 cells to fibronectin, collagen I, or laminin. **C**, inhibitory effect of emodin on spreading of MDA-MB-231 cells to fibronectin, collagen I, or laminin. **Top**, images of cell spreading on fibronectin, collagen I, laminin, or BSA-coated surface; **bottom**, quantitative data based on at least three independent experiments. **Columns**, mean of three independent experiments expressed as percentage comparing with the control without emodin treatment; **bars**, SD.

MDA-MB-231 cells (Fig. 1B). Furthermore, we also tested the effect of emodin on cell spreading, another common approach to assess cell adhesion. Results in Fig. 1C show that emodin significantly suppressed cell spreading on fibronectin, collagen, or laminin. With emodin treatment, most of the cells showed defects in polarized extension and remained with a rounded morphology.

Inhibitory effect of emodin on focal adhesion formation. Focal adhesion formation is an essential step in cell adhesion (26). In an effort to understand the mechanisms involved in the inhibitory effect of emodin on cell adhesion, we examined the effect of emodin on the distribution of FAK, paxillin, and vinculin, three major components of focal adhesions using immunofluorescence staining. As shown in Fig. 2A, focal adhesions were formed at both the central and the peripheral parts of cells after 60 minutes of attachment. In contrast, emodin treatment led to the reorganization of FAK, paxillin, and vinculin into granule-like or diffused pattern of staining. Meanwhile, consistent with the results in Fig. 1C, cells treated with emodin remained rounded with significantly reduced cell flattening and spreading.

Effect of emodin on ligand binding and expression level of integrins. The data presented above have shown clearly the inhibitory effect of emodin on focal adhesion formation. It is generally accepted that integrins mediate the focal adhesion formation and subsequent attachment of cells to ECM (5, 6).

Therefore, such inhibitory effect could be achieved via the following mechanisms: (a) direct interference on the integrin-ligand binding, (b) down-regulation of integrin expression level on cell surface, and (c) disruption on the integrin-mediated signaling pathway. Here, we first studied the effect of emodin on the functionality of integrin $\alpha_5\beta_1$ (fibronectin receptor) and $\alpha_1\beta_1$ (collagen I and IV and laminin receptor) using solid-phase binding assay with purified integrins. Minimal effect of emodin was observed on suppressing either integrin $\alpha_5\beta_1$ binding to fibronectin or integrin $\alpha_1\beta_1$ binding to collagen or laminin, respectively (data not shown). These data suggest that emodin has no significant effect on the binding of integrins and their ligands. Meanwhile, to verify the possible effect of emodin on integrin expression levels, we measured the surface expression of integrins by flow cytometry and found that emodin had no significant effect on the surface expression of integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_1\beta_1$ (data not shown).

Effects of emodin on the recruitment of FAK to integrins and formation of FAC. Because emodin has no significant effect on either integrin-ligand binding or surface expression of integrins, we decided to look into its possible effect on integrin-mediated signaling pathway. It is well reported that the recruitment of FAK to activated integrins is an early consequence of integrin-ligand interaction followed by rapid autophosphorylation of FAK-Tyr397 (5, 6, 27). Thus, we tested the association of FAK with integrin β_1 ,

a common integrin subunit involved in cell adhesion to fibronectin, collagen, and laminin. Immunoprecipitation analysis revealed that attachment to fibronectin significantly induced association of FAK with integrin β_1 (Fig. 2B). Treatment with emodin resulted in evident inhibition on the association of FAK with integrin β_1 in a time-dependent manner, suggesting that the recruitment of FAK to integrin β_1 was disrupted with emodin treatment. Similarly, when further examining the effect of emodin on the phosphorylation of FAK-Tyr³⁹⁷, an event following the recruitment of FAK to activated integrins (5, 6, 27), we found that the presence of fibronectin markedly enhanced FAK-Tyr³⁹⁷ phosphorylation. Emodin treatment caused a dramatic decrease in the phosphorylation level of FAK-Tyr³⁹⁷ in a time-dependent fashion (Fig. 2C).

Furthermore, we examined the effect of emodin on direct protein-protein interaction within FAC. As shown in Fig. 2D, there was an increased association of FAK with paxillin and vinculin after cell exposure to fibronectin. Emodin significantly inhibited the association of FAK with the other two focal adhesion molecules in a time-dependent manner, suggesting that FAC formation was disrupted with emodin treatment. To confirm the above finding, we did a reverse coimmunoprecipitation using anti-paxillin antibody. Emodin markedly suppressed the association of paxillin with FAK and vinculin in a dose-dependent manner (data not shown). Taken together, these results suggest that emodin might disrupt the integrin-mediated signaling cascade through inhibiting the recruitment of FAK to integrins and subsequent FAC formation.

Emodin inhibited the lipid raft clustering and subsequent association of integrin β_1 and FAC proteins with lipid rafts.

It is reported that the recruitment of FAK to integrins is the consequence of integrin clustering induced by interaction with specific ligands (5, 27). Thus, to further explore the underlying mechanism involved, we decided to look into the possible effect of emodin on integrin clustering. Recently, lipid raft, a microdomain rich in sphingolipids and cholesterol, has been increasingly recognized to play a critical role in modulating the integrin clustering and functions through raft coalescence mechanism (9, 28). Therefore, in this study, we first used confocal microscopy to validate whether there is lipid raft and integrin clustering as well as colocalization of lipid rafts and integrin β_1 on cell-matrix interaction. Owing to its highly specific binding to raft ganglioside GMI, Alexa Flour 488-conjugated CTxB was used to visualize membrane rafts (20). Meanwhile, cells were costained with anti-integrin β_1 antibody (in red fluorescence). The results in Fig. 3A show that cell attachment to fibronectin quickly led to significant coalescence of lipid rafts into distinct larger patches. At the same time, clustering of integrin β_1 was also observed. The colocalization of integrin β_1 with lipid raft patches happened within 10 minutes after seeding and started to decrease after 20 minutes. These results show the rapid association of integrin β_1 with membrane rafts after cell exposure to fibronectin.

Based on these observations, we subsequently tested whether emodin has any effect on the raft and integrin β_1 clustering and

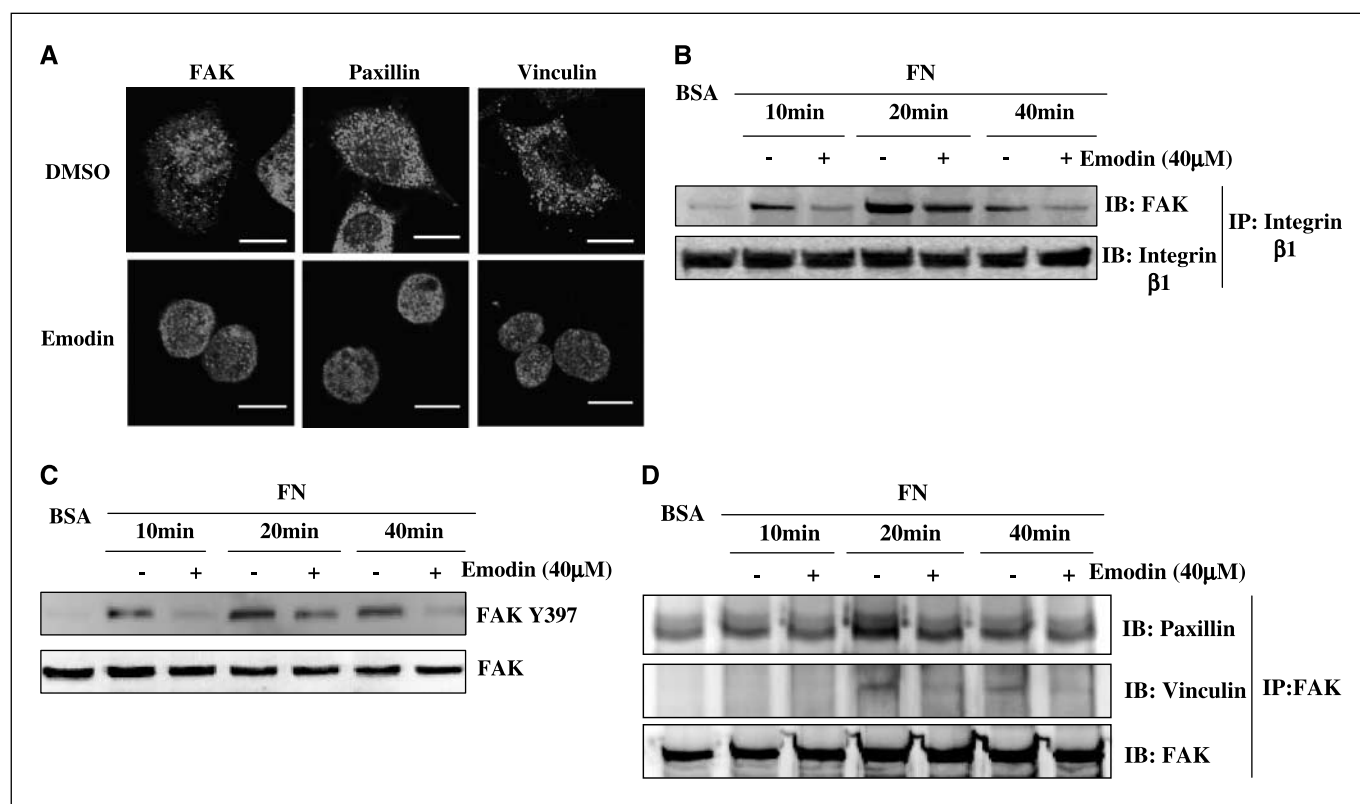


Figure 2. Emodin suppresses focal adhesion formation, recruitment of FAK to integrins, and formation of FAC. **A**, MDA-MB-231 cells were pretreated with or without 40 $\mu\text{mol/L}$ emodin for 1 hour and seeded onto fibronectin-coated coverslips for 1 hour. Cells were then fixed and immunostained with anti-FAK, anti-paxillin, or anti-vinculin antibodies as described in Materials and Methods. Images were examined and photographed by confocal microscopy. Bars, 20 μm . **B**, cells were pretreated with or without emodin for 1 hour and seeded onto Petri dishes coated with fibronectin or BSA. Cell lysates were immunoprecipitated with antibody against integrin β_1 (IP: *Integrin- β_1*) and immunoblotted with antibody against FAK (IB: *FAK*) after cells were seeded for designated period. **C**, cells were treated the same as in (B). Whole-cell lysates were subjected to immunoblotting with antibodies against phosphorylated Y397 FAK and FAK. **D**, cell lysates were immunoprecipitated with an anti-FAK antibody (IP: *FAK*) and immunoblotted with antibody against paxillin (IB: *Paxillin*) and vinculin (IB: *Vinculin*).

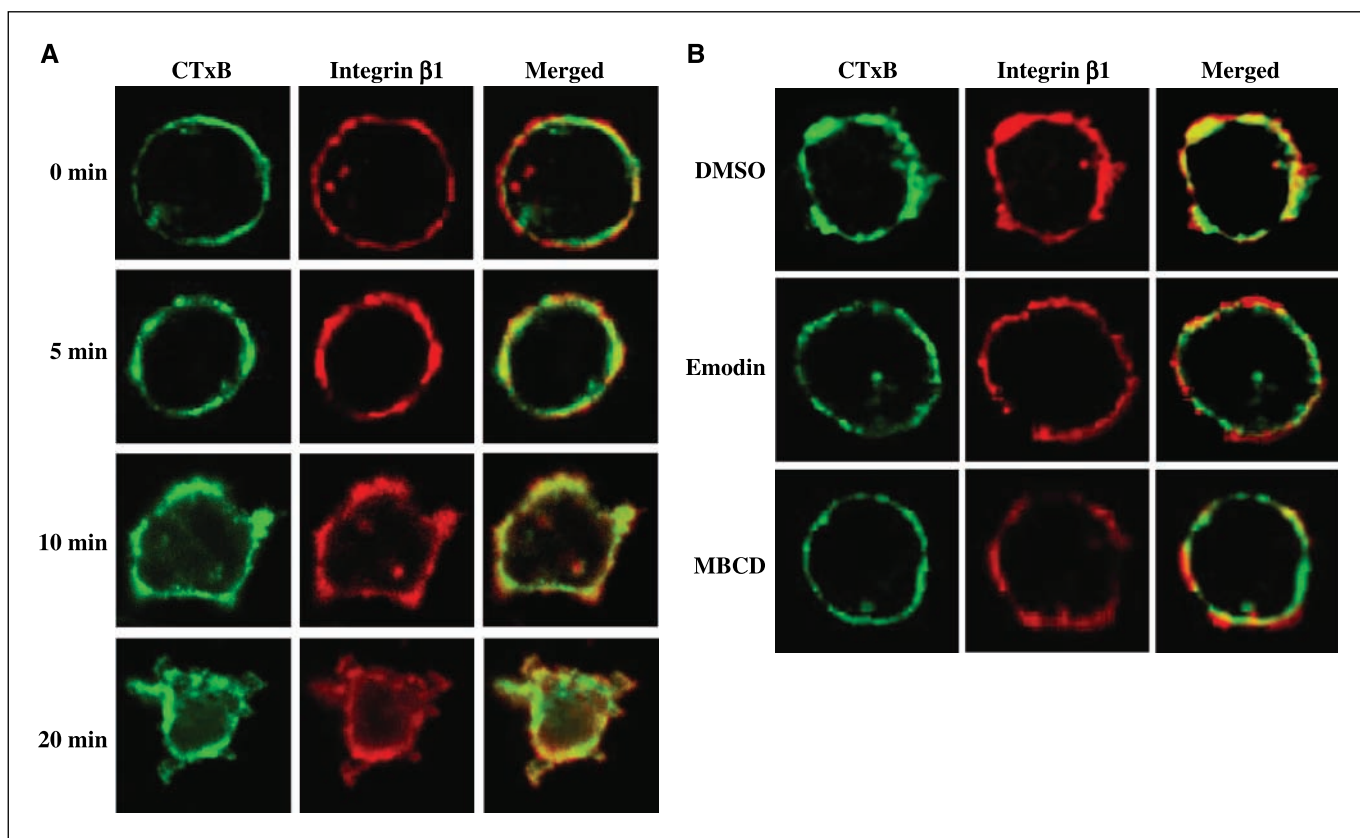


Figure 3. Emodin inhibits the lipid rafts and integrin clustering and the colocalization of integrin β_1 with lipid rafts. *A*, MDA-MB-231 cells were fixed at 0, 5, 10, and 20 minutes after seeding onto fibronectin-coated surface. Cells were costained with Alexa Fluor 488-conjugated CTxB (green) and anti-integrin β_1 (red). *B*, cells were pretreated with 40 $\mu\text{mol/L}$ emodin or 2 mmol/L MBCD for 1 hour and seeded onto fibronectin-coated coverslips for 10 minutes followed by immunostaining with Alexa Fluor 488-conjugated CTxB (green) and integrin β_1 antibody (red). Cells were analyzed by confocal microscopy. Representative cell for each treatment. Magnification, $\times 600$.

colocalization of integrin β_1 and lipid rafts 10 minutes after seeding, the moment with maximum colocalization. As shown in Fig. 3*B*, emodin markedly suppressed integrin β_1 clustering and colocalization of integrin β_1 with raft patches. Another interesting finding is that emodin inhibited the lipid raft clustering similar to that of MBCD, a known inhibitor of lipid raft formation (29). These results are consistent with the data in Fig. 2 and suggest that emodin might affect raft and integrin clustering followed by inhibiting FAK formation and cell adhesion.

To further verify this hypothesis, we next examined the functional association of lipid raft, integrins, and FAK molecules using nonionic detergent fractionation methods. Membrane rafts are microdomains of the plasma membrane enriched with cholesterol and sphingolipids. They are insoluble after treatment with nonionic detergents, such as Triton X-100, at 4°C (7). We first examined the distribution of integrin β_1 between the detergent-soluble and detergent-insoluble fractions of MDA-MB-231 cells. Integrin β_1 started to transfer from the nonraft (soluble) fraction to the raft (insoluble) fraction shortly 5 minutes after seeding and returned to nonraft (soluble) fraction 10 minutes afterward (Fig. 4*A*). Meanwhile, FAK, paxillin, and vinculin were found to translocate from the soluble fraction to the insoluble fraction similar to that of integrin β_1 . However, their translocation lasts much longer. These results suggest that lipid rafts are essential in integrin-mediated signaling, and exposure to fibronectin induces the translocation of integrin β_1 followed by FAK proteins from

nonrafts to rafts on the membrane. Besides, it is reported that c-Src is associated exclusively with membrane rafts, and Tfr could serve as a nonraft marker (30). Here, we found that substantial amount of c-Src mainly existed in the insoluble fraction, and Tfr was hardly detectable in the insoluble fraction (Fig. 4*A*). This further confirms the quality of the sample preparation in this study.

Based on these observations, we next examined the effect of emodin on the distribution of integrin β_1 and FAK molecules 10 minutes after seeding. Emodin and MBCD markedly suppressed the translocation of integrin β_1 , FAK, paxillin, and vinculin from the soluble fraction to insoluble fraction after seeding (Fig. 4*B*). Taken together with the data in Fig. 3*B*, these results confirm that the inhibition of emodin on cell adhesion is through its suppression on lipid raft clustering and subsequent colocalization of integrins and focal adhesion molecules with rafts.

Effect of emodin on the change of cholesterol level and lipid profile. To further explore the disruptive effect of emodin on lipid rafts, we explored whether emodin would alter the lipid profiling of membrane rafts. Cholesterol is one of the major components of lipid rafts (28). In this study, the APCI/MS analysis shows that the cholesterol content in the insoluble fraction decreased by $\sim 16\%$ in the emodin-treated cells compared with the control group, similar to the effect of MBCD (Fig. 5*A*). Whole-cell cholesterol content remained the same with or without emodin treatment (data not shown). These results suggest that emodin treatment leads to evident decrease of cholesterol in the raft fraction.

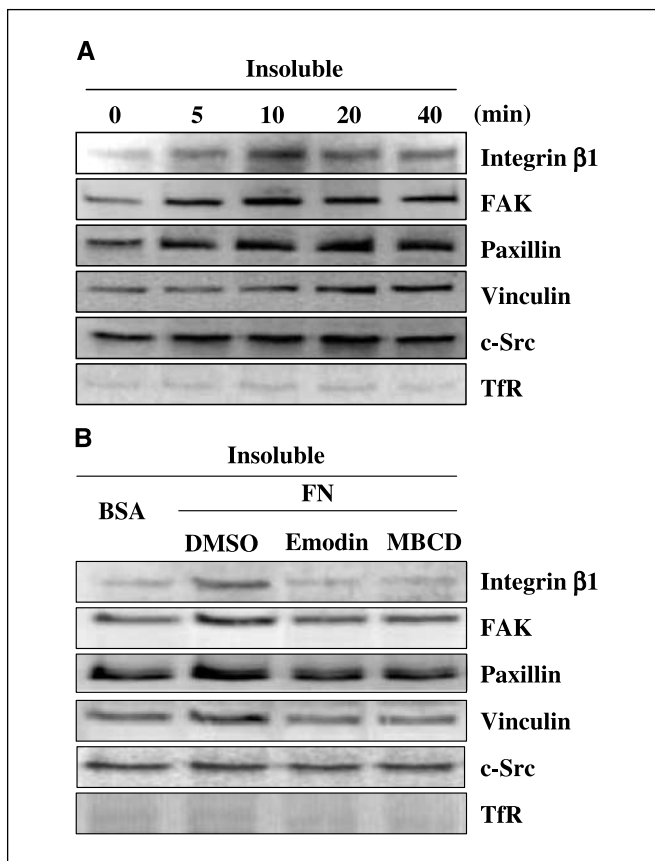


Figure 4. Emodin suppresses the association of integrin β_1 and FAK molecules with membrane lipid rafts. *A*, MDA-MB-231 cells were seeded onto fibronectin-coated Petri dishes for up to 40 minutes. Detergent-insoluble (proteins rich in membrane raft) fractions of cell lysates were separated as described in Materials and Methods and subjected to 10% SDS-PAGE and immunoblotted with anti-integrin β_1 , FAK, paxillin, vinculin, c-Src, and Tfr antibodies, respectively. Representative of three independent experiments. *B*, cells were first treated with 40 $\mu\text{mol/L}$ emodin or 2 mmol/L MBCD for 1 hour and then seeded onto fibronectin- or BSA-coated Petri dishes for 10 minutes. Detergent-insoluble fractions of cell lysates were subjected to SDS-PAGE and immunoblotted with anti-integrin β_1 , FAK, paxillin, vinculin, c-Src, and Tfr antibodies, respectively.

Because sphingolipids are another critical component of lipid rafts (28), we also examined the effect of emodin on lipid profile. Figure 5B shows the ESI/MS profiles of lipid extracts from detergent-insoluble fractions. Emodin induced remarkable decrease of various major sphingolipids in the insoluble fractions, including sphingomyelin 18/24:0, sphingomyelin 18/16:0, ceramide glycoside 18/24:0, ceramide 18/16:0, ceramide 18/24:0, and ceramide 18/24:1. Similar decrease was observed after MBCD treatment (data not shown).

Cholesterol replenishment restored the translocation of integrin β_1 and FAK molecules to lipid raft and cell adhesion inhibited by emodin. To further confirm the involvement of cholesterol in the inhibitory effect of emodin on cell adhesion, we did the cholesterol replenishment experiment. As shown in Fig. 5A, cholesterol replenishment (30 $\mu\text{g/mL}$) completely restored the cholesterol level of raft fraction in cells treated with emodin or MBCD. It was also observed that cholesterol replenishment effectively restored the sphingolipid decrease induced by emodin (Fig. 5B) or MBCD (data not shown). These data suggest that emodin induced cholesterol-dependent decrease of sphingolipids in the lipid raft structure.

Moreover, cholesterol replenishment totally abolished the inhibitory effect of emodin as well as MBCD on the translocation of integrin β_1 and FAK molecules to lipid raft (Fig. 6A), suggesting that emodin is likely to disrupt the integrin signaling pathway by induction of cholesterol reduction in lipid raft. Lastly and more importantly, cholesterol replenishment was able to prevent the decrease of cell adhesion induced by emodin (Fig. 6B). Taken together, these findings suggest that emodin is likely to target on the lipid raft cholesterol and subsequently disrupt lipid raft clustering, which leads to inhibition of FAK formation and finally prevents cancer cell adhesion.

Discussion

Cell adhesion, together with cell migration and invasion, is an essential biological process that is involved in cancer metastasis (2). Interruption of cell adhesion has been considered as an important strategy for prevention and treatment of cancer metastasis. In this study, we characterized the inhibitory effect of emodin on adhesion of various human cancer cells. This inhibition

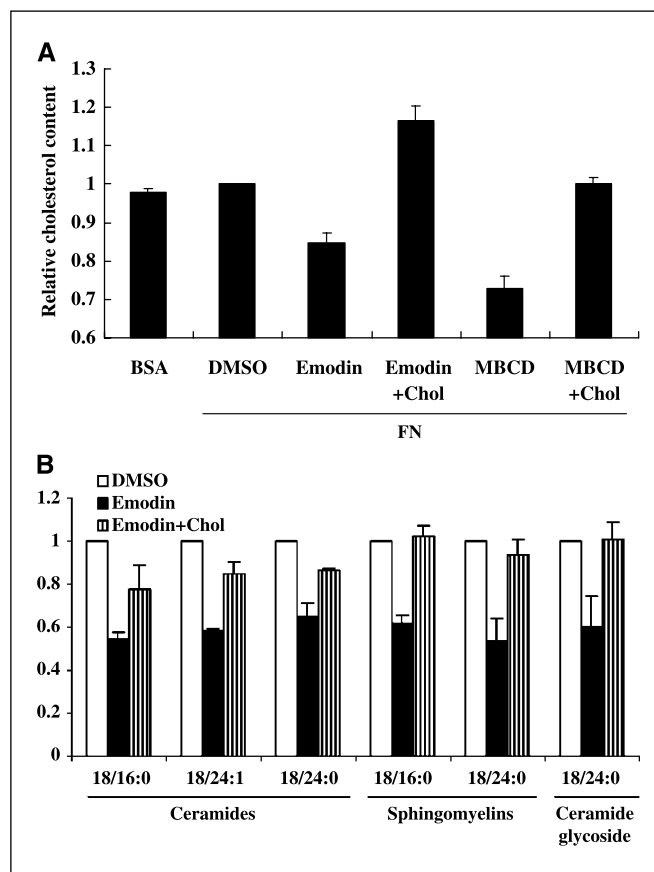


Figure 5. Emodin reduces the cholesterol and sphingolipids content in lipid rafts. MDA-MB-231 cells were treated with 40 $\mu\text{mol/L}$ emodin or 2 mmol/L MBCD for 1 hour followed by 30 minutes of cholesterol replenishment (30 $\mu\text{g/mL}$). Cells were then planted onto fibronectin-coated Petri dishes for 10 minutes. Detergent-insoluble fractions of cell lysates were separated and subjected to lipid profile analysis as described in Materials and Methods. Data are relative content compared with DMSO control group. *A*, lipids of detergent-insoluble fractions were extracted and subjected to APCI/MS analysis of cholesterol content. *B*, change of sphingolipids in insoluble fraction after emodin treatment with (Emodin+Chol) or without cholesterol replenishment. Columns, mean of three independent experiments; bars, SD.

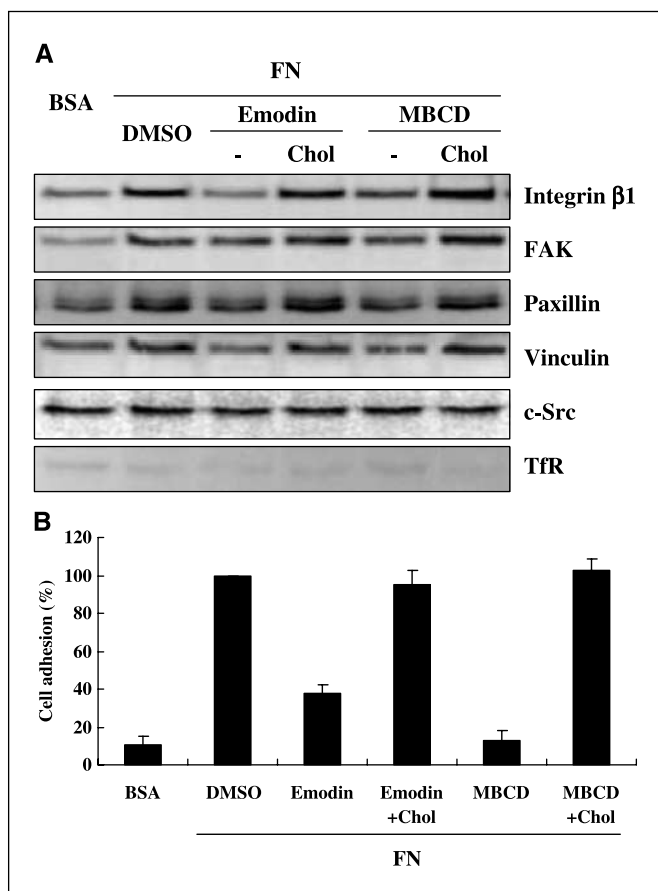


Figure 6. Cholesterol replenishment prevents the adverse effect of emodin on lipid rafts, integrin signaling, and cell adhesion. MDA-MB-231 cells were treated with 40 $\mu\text{mol/L}$ emodin or 2 mmol/L MBCD for 1 hour followed by 30 minutes of cholesterol replenishment (30 $\mu\text{g/mL}$). Cells were then planted onto fibronectin-coated Petri dishes for 10 minutes. *A*, detergent-insoluble fractions were subjected to 10% SDS-PAGE and immunoblotted with anti-integrin β_1 , FAK, paxillin, vinculin, c-Src, or TfR antibodies, respectively. *B*, cell adhesion assay was conducted as described in Materials and Methods. Columns, mean of three independent experiments and expressed as percentage of DMSO control group; bars, SD.

is likely due to its disruptive effect on the components of membrane lipid rafts, thus inhibiting the lipid raft-associated integrin signaling pathway.

It is well established that once the malignant cells have detached from the primary tumor they might reach an ectopic site and adhere to the meshwork of ECM, which contains various components, such as fibronectin, collagen, and laminin. Failed attachment of malignant cells at a new site might frequently lead to the immediate initiation of apoptosis (31). In this study, emodin displays potent inhibitory effect on cell adhesion in various cancer cells, suggesting that this inhibition is not cell type specific. Notably, our results also showed that emodin inhibits cell adhesion not only to fibronectin but also collagen I and laminin, suggesting that this inhibitory potential applies to a broad spectrum of ECM. At present, several cell adhesion inhibitors have been actively investigated, such as gefitinib (32) and inositol hexaphosphate (33). Furthermore, several cyclic peptide inhibitors and antibodies, which block cell-matrix interaction, such as cilengitide and vitaxin, are now being tested in clinical phase I/II trials (34). Therefore, taken together with our previous findings on potent inhibitory effect of emodin on cancer cell invasion (18) and

migration (17), emodin seems to be a strong candidate for developing as a therapeutic agent to prevent cancer metastasis.

Integrins are a large family of adhesion molecules on cell membrane responsible for cell attachment to ECM proteins. The interactions between integrin and ECM result in signaling cascades, which promote both cell adhesion and spreading. Abnormalities and improper localization of these integrins at the cell surface have been observed in mammalian cancer cells and are believed to be closely related to their metastatic behavior (35, 36). Because emodin does not directly affect integrin-ligand binding and surface integrin expression, we turned our attention to the possible effect of emodin on the integrin-initiated signaling pathway. There are several key steps involved in this signaling, including integrin receptor clustering or oligomerization. Recent evidence suggests that integrin clustering and functioning is regulated by their recruitment into discrete membrane rafts, which are sphingolipid- and cholesterol-rich microdomains (10). One mode by which rafts transmit signals involves their coalescence into large platforms into which signaling proteins translocate and concentrate (37). Consistently, in the present study, after exposure to fibronectin, small membrane rafts were observed to coalesce into larger patches, whereas emodin inhibited this process to block the colocalization of integrin β_1 with lipid rafts (Figs. 3 and 4). The newly created larger rafts would help to assemble different proteins to facilitate the signaling pathway. Thus, the inhibition of lipid raft clustering by emodin might be the underlying mechanism leading to the suppression of integrin clustering, FAC formation, and eventually cell adhesion. Although the molecular mechanisms of integrin-lipid raft interactions are yet unclear, both cholesterol and sphingolipids, two essential components of lipid rafts, have been reported to play specific roles in regulating the integrin functions (9). Therefore, to further explore the mechanism involved in the disruptive effect of emodin on lipid raft, we turned our focus onto the possible changes of cholesterol and sphingolipids within lipid rafts.

As a neutral lipid, cholesterol accumulates in lipid rafts (28). The hydrogen bonding between 3'-OH group of cholesterol and amide group of sphingolipids contributes to the formation of sphingolipid-cholesterol liquid-ordered domains (38). Reduction of the cholesterol concentration in the plasma membrane can lead to the loss of signaling function of lipid rafts (39). Our results showed that emodin decreases the cholesterol level in the detergent-insoluble fractions and consequently suppresses lipid raft coalescence and cell adhesion (Figs. 3 and 5). Although it is not clear how emodin causes cholesterol reduction in insoluble fraction of plasma membrane, one possible explanation is that emodin has been shown to reside at the upper half of the phospholipid hydrocarbon chains in model membranes (40), thus affecting the tight interaction between cholesterol and sphingomyelins and finally excluding cholesterol from lipid rafts.

On the other hand, sphingolipid, typified by sphingomyelin, is one of the major classes of membrane phospholipids (41). Several reports have highlighted that sphingolipids are associated with integrins and are capable of modulating their activity (9, 42). Besides, ceramide, a major metabolic product of sphingomyelins, is reported to increase the tight packing of the acyl chains in the bilayer, promote stabilization of raft formation, and provide the driving force for coalescence of rafts into platforms (43, 44). In the present study, sphingomyelins, ceramides, and ceramide glucosides were found to be decreased in the lipid raft fraction after emodin treatment (Fig. 5B). These decreases might directly affect the tight

packing and stabilization of lipid rafts, thus impairing the signaling function of lipid rafts.

Furthermore, our cholesterol replenishment results confirmed that cholesterol contributes to the inhibition of cell adhesion by emodin. Replenishment of cholesterol restored not only the cholesterol level in the lipid rafts (Fig. 5A) but also the content of sphingomyelins and ceramides decreased by emodin (Fig. 5B). This suggests that emodin-induced reduction of these sphingolipids in membrane rafts is likely to be the result of the disorganization in the lipid raft domains brought by the removal of cholesterol. Meanwhile, cholesterol replenishment also recovers the translocation of integrin β_1 and FAC molecules into raft fraction (Fig. 6A) and eventually reverses the inhibitory effect of emodin on cell adhesion (Fig. 6B). These findings further show that the decrease of cholesterol in lipid rafts is critical in mediating the disruptive effect of emodin on the lipid raft-associated integrin signaling and cell adhesion. There is a possibility that, through this particular mechanism, emodin may also affect the function of other membrane proteins that are lipid raft associated, although further investigations are needed to confirm this. Nevertheless, recent studies suggest that the characteristics of membrane lipids of cancer and noncancer cells are rather different, which lead to our speculation that emodin might have higher specificity on cancer cells. For example, relative higher content of cholesterol and saturated fatty acids, including sphingolipids, was found in cancer

cells (45, 46). Moreover, in cancer cells, the structure and function of lipid rafts may be modified in such a way that it would enhance cancer cell survival (47). Thus, in this case, due to its disruptive effect on cholesterol in the lipid rafts, emodin might have a stronger inhibitory effect on cancer cells than normal cells.

Collectively, in this study, we present novel evidence that emodin displays significant inhibitory effect on cell adhesion in various cancer cells. Such inhibitory effect by emodin is through cholesterol decrease in lipid rafts and subsequent suppression of the lipid raft-associated integrin signaling pathway. Emodin has been reported previously to possess strong effect against tumor cell migration (17) and invasion (18). Taken together, these findings suggest that emodin, which potentially interferes with tumor metastasis at all three critical steps, could be of therapeutic value in preventing metastasis of human cancers.

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