Curcumin Inhibition of Integrin (α6β4)-Dependent Breast Cancer Cell Motility and Invasion

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Abstract  Curcumin, a polyphenol natural product isolated from the rhizome of the plant *Curcuma longa*, has emerged as a promising anticancer therapeutic agent. However, the mechanism by which curcumin inhibits cancer cell functions such as cell growth, survival, and cell motility is largely unknown. We explored whether curcumin affects the function of integrin α6β4, a laminin adhesion receptor with an established role in invasion and migration of cancer cells. Here we show that curcumin significantly reduced α6β4-dependent breast cancer cell motility and invasion in a concentration-dependent manner without affecting apoptosis in MDA-MB-435/β4 (β4-integrin transfectants) and MDA-MB-231 breast cancer cell lines. Further, curcumin selectively reduced the basal phosphorylation of β4 integrin (Y1494), which has been reported to be essential in mediating α6β4-dependent phosphatidylinositol 3-kinase activation and cell motility. Consistent with this finding, curcumin also blocked α6β4-dependent Akt activation and expression of the cell motility–promoting factor ENPP2 in MDA-MB-435/β4 cell line. A multimodality approach using curcumin in combination with other pharmacologic inhibitors of α6β4 signaling pathways showed an additive effect to block breast cancer cell motility and invasion. Taken together, these findings show that curcumin inhibits breast cancer cell motility and invasion by directly inhibiting the function of α6β4 integrin, and suggest that curcumin can serve as an effective therapeutic agent in tumors that overexpress α6β4.

In this study, we tested the effect of curcumin treatment on the function of a well-known tumor antigen, α6β4 integrin. Initially, α6β4 has been characterized as a receptor for laminin family members of extracellular matrix. It mediates hemidesmosome formation and tissue integrity in normal epithelia where it serves as an adhesion receptor (18). In aggressive cancer cells, however, the host-tumor microenvironment induces mobilization of α6β4 from hemidesmosome into filamentous actin–based structures such as lamellipodia and filopodia, where this integrin becomes signaling competent by functionally interacting with other growth factor receptors and G-protein–coupled receptors (19). The enhanced signaling function of α6β4 contributes to the malignant behaviors of cancer cells. For example, the level of α6β4 expression in breast carcinoma cells correlates with their ability to invade (20, 21) and to survive under conditions of nutrition deprivation (22, 23). This is associated with its activation of the phosphatidylinositol 3-kinase (PI3K)-Akt (protein kinase B) signaling pathway (20, 22, 24), which is critical for cell proliferation, growth, survival, and migration.

In this study, we tested whether curcumin inhibits α6β4 signaling and functions important for breast cancer cell motility and invasion using MDA-MB-435/β4 and MDA-MB-231 breast cancer cells because curcumin has been shown to inhibit Akt (11, 24) and nuclear factor kB (25, 26), both of which are known downstream effectors of α6β4 (20, 27). We found that curcumin inhibits α6β4-dependent cancer cell motility and invasion by reducing the key tyrosine residue (Y1494) phosphorylation of the β4-integrin cytoplasmic tail as well...
as phosphorylation of Akt, a downstream target of αβ4. Reduction of cell motility and invasion by curcumin also correlates with the reduction in expression of ENPP2, which is the key cell motility–promoting factor whose expression is selectively enhanced by αβ4 (28). Curcumin also works cooperatively with other pharmacologic inhibitors known to block αβ4 signaling pathways at lower concentrations. All together, our studies present the first evidence of curcumin inhibition of integrin function, which leads to the blockade of integrin-dependent cancer cell motility and invasion.

Materials and Methods

Cell lines and reagents

The MDA-MB-435 and MDA-MB-231 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University (Washington, DC). The generation of MDA-MB-435 subclones (MDA-MB-435/mock (vector only) and MDA-MB-435/α4 (β4 integrin) was done as previously described (20, 22, 29). MDA-MB-231 cells were stably infected with lentiviruses that expressed shRNA targeted against either green fluorescent protein or the β4-integrin subunit as previously described (30). These cells were cultured in low-glucose DMEM with L-glutamine, sodium pyruvate, 10% fetal bovine serum, and 100 units/mL of penicillin and streptomycin. For inhibitor pretreatment, cells were preincubated with the indicated doses (see figure legends) of curcumin (Sigma), SU11274 (Calbiochem), and Akt inhibitor (Calbiochem) for either 3 or 12 h before the assays. Integrin β4 (clone H-101) and actin (clone C-11) antibodies were purchased from Santa Cruz Biotechnology, and Akt and p-Akt (Ser473 and Thr383) antibodies were obtained from Cell Signaling Technology. Phospho-β4-integrin (Y1494) was obtained from ECM Biosciences.

Cell motility and invasion assays

For the cell motility assay, the upper and lower surfaces of the membrane in transwell inserts (Costar) were coated with collagen I at 4°C overnight. To prepare for the invasion assay, Matrigel (Collaborative Research; 0.5 μg) was diluted with cold water and dried onto each filter overnight at room temperature. On the following day, transwell membranes were blocked with DMEM for 1 h at 37°C. Cells were trypsinized and resuspended in serum-free DMEM/bovine serum albumin. A total of 105 cells were added to the upper chamber of each well. Lysophosphatidic acid (LPA; 100 μmol/L) was added to the lower chambers as a chemotaxtractant. Inserts were incubated for 2 to 3 h, and nonmigrating cells were mechanically removed with cotton swabs. The cells attached to the bottom side of the membrane were stained with crystal violet and counted. Assays were done in triplicate and repeated five times. Cell motility and invasion were quantified by counting the cells that migrated to the lower surface of the membrane per square milliliter using bright-field optics. Data represent the mean ± SD and are pooled from five independent experiments.

Three-dimensional matrix culture

Three-dimensional culture matrix obtained from growth factor-reduced basement membrane extract (RGF BME) was prepared as described in the manufacturer’s protocols (TreVigen, Inc.). Briefly, three-dimensional culture matrix was thawed and added to the desired culture plates and incubated at 37°C for 1 h to promote gelting of matrix. Assay medium (2% three-dimensional culture matrix RGF BME in tissue culture media) was prepared to dilute cells. A given number of harvested cells were added to the well of the plate containing three-dimensional culture matrix RGF BME and incubated at 37°C in a CO2 incubator overnight. Each day, cell growth and structure formation were monitored via an inverted microscope. Assay media were replaced every 3 d.

Western blot analysis

For the analysis of protein expression, cells were lysed in radioimmunoprecipitation assay (RIPA)-EDTA buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mmol/L EDTA] containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, and protease inhibitor. The lysate was clarified by centrifugation at 4°C. Equal amounts of extracts were resolved in SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). The rest of the Western blot analysis was done as described previously (22).

Apoptosis assay

Cells were incubated in 10% fetal bovine serum–containing DMEM for 12 h at 37°C with or without treatment at the indicated dose of curcumin. Subsequently, both adherent and nonadherent cells were harvested and apoptosis was measured using the Annexin V-PE Apoptosis Detection Kit 1 (BD Biosciences) as described previously (22). The data represent the mean ± SD and are pooled from three independent experiments.

Quantitative real-time PCR

Quantitative analysis of ENPP2 mRNA expression was done with real-time PCR using the ABI Prism 7700 sequence detection system as described by the manufacturer (Applied Biosystems). The of primers and probes are as follows: ENPP2 forward sequences (5′-ATGACATTCAGATCTGCA-3′; ENPP2 reverse primer, 5′-CTCACGATTTATACATGC3′; ENPP2 probe, 5′-GCTGATGCATATCTC-3′; β-actin forward primer, 5′-TCACAGATCATGATGATGC-3′; β-actin reverse primer, 5′-AAGCGCTTTCGCACT-3′; and β-actin probe, 5′-CGCTCGGCTAGCT-3′). The data represent the mean ratio of ENPP2 to β-actin mRNA ±SD obtained from triplicate samples.

Results

Curcumin inhibits αβ4-dependent breast cancer cell motility in a dose-dependent manner

Whereas a number of studies have shown that curcumin inhibits the motility of cells derived from hepatocellular carcinoma (31), rhabdomyosarcoma (32), colon (33), and prostate cancer (34), little is known about the mechanism by which curcumin accomplishes this. Based on our previous studies and others of those showing that αβ4 is critical for cell motility, we hypothesized that curcumin targets αβ4 signaling. To test this hypothesis, MDA-MB-231 and MDA-MB-435 breast cancer cell lines were used. MDA-MB-231 cells are highly motile and an invasive breast carcinoma cell line that endogenously expresses high levels of α4. The MDA-MB-435 cell line lacks endogenous expression of β4 integrin. Therefore, the stable clones that express β4 integrin by transfection (αβ4 positive) and null vector (mock control, αβ4 negative) were used for our studies. The motility of the cells was determined with a transwell assay.

As reported previously (20, 21, 35), the ability of these cells to migrate toward a chemotaxtractant such as LPA correlates with the level of αβ4 expression (Fig. 1). Briefly, expression of β4 integrin in MDA-MB-435 cells increased the cell motility by ∼7-fold (Fig. 1A), whereas a reduction of β4 integrin expression in MDA-MB-231 cells by shRNA decreased the cell motility by ∼40% (Fig. 1B). In both of the αβ4-positive cell lines (MDA-MB-435 β4 transfectants and MDA-MB-231 green fluorescent protein shRNA infectants), curcumin effectively blocked αβ4-dependent cell motility in a dose-dependent manner (Fig. 1). Significant inhibition of cell motility was
observed beginning at a dose of 5 μmol/L curcumin, and cell motility was almost completely blocked at 20 μmol/L (Fig. 1).

α6β4-null MDA-MB-435 mock transfectants were barely migratory even in the presence of LPA, which means that we did not see a dramatic inhibitory effect of curcumin either (Fig. 1A). On the other hand, we observed that down-regulation of β4-integrin expression by 70% (via densitometric analysis of Western blot data, Fig. 1B) with shRNA did not completely block their cell motility toward LPA in MDA-MB-231 cells.

Although curcumin effectively blocked cell motility in both MDA-MB-231 green fluorescent protein and β4-integrin shRNA expressing cells (Fig. 1B), it is interesting to note that its inhibitory effect is less effective in β4-integrin shRNA expressing cells. For example, treatment with 10 μmol/L curcumin led to a 77% reduction in motility of green fluorescent protein shRNA expressing MDA-MB-231 cells compared with that of control cells (without curcumin treatment), whereas it blocked 56% of motility of β4-integrin shRNA expressing MDA-MB-231 compared with that of control cells (Fig. 1B). These results suggest that curcumin may target α6β4 signaling to inhibit cell motility, and α6β4 may sensitize breast cancer cells to curcumin treatment.

**Curcumin prevents the α6β4-dependent invasive phenotype of breast cancer cells**

Cell motility is an essential component of the invasive phenotype of cancer cells. To obtain more conclusive evidence to determine whether curcumin could blunt breast cancer cell invasion, we used three-dimensional culture matrix systems that provide growth factor–reduced Matrigel to mimic the matrix environments breast cancer cells encounter in vivo (Fig. 2A). Expression of α6β4 in MDA-MB-435 cells induced a dramatic neomorphic effect, producing protrusive extensions that invaded basement membrane gels (Fig. 2A). Treatment with curcumin efficiently blocked α6β4-dependent protrusive extension as well as the growth of MDA-MB-435 cells under three-dimensional Matrigel culture (Fig. 2A). Transwell-based invasion assays further confirmed that curcumin blocked the invasion of the three α6β4-positive breast cancer cell lines (MDA-MB-435 β4 transfectants, MDA-MB-231, and SUM-159) in a dose-dependent manner (Fig. 2B). The invasion of these cell lines was previously shown to depend on α6β4 (20, 21, 35).

**Curcumin inhibition of α6β4-dependent breast cancer cell motility and invasion is not due to apoptosis**

Considering previous reports that curcumin induces apoptosis of cancer cells depending on the cancer cell type and the dosage of curcumin treatment (16, 24, 36), one concern raised in relation to the cell motility and invasion assays is whether the inhibitory effect of curcumin is related to apoptosis. To address this issue, we conducted an apoptosis assay in MDA-MB-435 β4-integrin transfectants (Fig. 3A) and MDA-MB-231 cells (Fig. 3B) with the Annexin V-PE Apoptosis Detection Kit. As shown in Fig. 3, there was no significant increase in...
apoptosis in either of these cell lines when treated with doses of curcumin from 5 to 20 μmol/L, within which range we saw an inhibitory effect on cell motility and invasion (Fig. 1). We did observe a detectable increase in apoptosis in these cells on curcumin treatment at concentrations ≥30 μmol/L (Fig. 3). Therefore, we concluded that curcumin inhibits cell motility and invasion at relatively low concentrations (5-20 μmol/L) and that this inhibitory effect is not due to its induction of apoptosis.

**Curcumin inhibits the phosphorylation of a key tyrosine residue of the β4 subunit and α6β4 signaling cascades important for cell motility**

To address the mechanism by which curcumin inhibits α6β4 functions such as cell motility and invasion, we assessed whether the inhibition of α6β4 occurs directly at the receptor level. A tyrosine residue (Y1494) in the third fibronectin type III domain of the β4 cytoplasmic tail has been shown to be essential for initiating α6β4-dependent signaling cascades to promote carcinoma invasion and survival (22, 29). The level of Y1494 phosphorylation is also an indication of the signaling competency of α6β4 (29). Therefore, we tested whether curcumin affects the phosphorylation of this key tyrosine residue of β4 integrin. As shown in Fig. 4A, the basal phosphorylation level of Y1494 was dramatically reduced by even as low as 5 μmol/L curcumin, suggesting that curcumin directly inhibits α6β4.

Based on a previous report that α6β4 enhances cell motility and invasion through activation of the PI-3K/Akt pathway (20, 24, 35) and up-regulation of cell motility–promoting factors such as ENPP2 (28), we assessed the effect of curcumin on these downstream signaling events of α6β4. Our results indicate that curcumin effectively blocked α6β4-dependent phosphorylation of Akt at Ser473 and Thr308 (indication of Akt activity) and up-regulation of ENPP2 as assessed by Western blot analysis (Fig. 4B). The data suggest that curcumin directly inhibits α6β4 function and subsequently blocks downstream targets of α6β4. Using quantitative real-time PCR, we further confirmed that curcumin inhibited α6β4-dependent ENPP2 expression, suggesting that ENPP2 expression is regulated by α6β4 and curcumin at the mRNA level (Fig. 4C).
The multimodality approach using curcumin with pharmacologic inhibitors of c-Met and Akt enhances inhibition of α<sub>6</sub>β<sub>4</sub>-dependent breast cancer cell motility and invasion

Because the combination of chemotherapeutic agents generally results in greater tumor suppressive responses and fewer side effects in cancer patients, we investigated multimodality approaches by combining curcumin with other pharmacologic inhibitors to see whether additive effects occurred in the inhibition of α<sub>6</sub>β<sub>4</sub>-dependent cancer cell motility and invasion. We chose a pharmacologic inhibitor against c-Met, the HGF receptor tyrosine kinase, because α<sub>6</sub>β<sub>4</sub> has been shown to functionally interact with c-Met (35, 37). Another target that we chose is Akt, a well-known downstream effector of α<sub>6</sub>β<sub>4</sub> (20, 29). Inhibition of c-Met activity by the c-Met specific inhibitor SU11274 at 5 μmol/L reduced α<sub>6</sub>β<sub>4</sub>-dependent cell motility by ∼45%, which is similar to its inhibition by 5 μmol/L curcumin in MDA-MB-435 β<sub>4</sub> transfectants (Fig. 5A). However, when these two compounds were used in combination at the above concentration, the inhibitory effect increased to 75% (Fig. 5A). When curcumin was combined with Akt inhibitor, the inhibitory effect increased to 95% (Fig. 5A). The additive effect of curcumin with either SU11274 or Akt inhibitor was also observed to prevent the invasive phenotype of MDA-MB-453 cells in three-dimensional matrix culture (Fig. 5B).

Whereas combining curcumin with SU11274 or Akt inhibitor at the indicated dose (Fig. 5) dramatically increased the inhibitory effect on cell motility and invasion, there was no significant increase in apoptosis (data not shown), suggesting that curcumin inhibits the phosphorylation of a key tyrosine residue of the β<sub>4</sub> subunit and α<sub>6</sub>β<sub>4</sub> signaling cascades important for cell motility. A, MDA-MB-435 β<sub>4</sub> transfectants were pretreated with or without the indicated doses of curcumin for 3 h before lysis with RIPA buffer. Extracts from these cells were analyzed for Western blot analysis with antibodies against β<sub>4</sub>-integrin, phospho-β<sub>4</sub>-integrin (Y1494), and β-actin. B, MDA-MB-435 mock and β<sub>4</sub> transfectants were pretreated with or without the indicated doses of curcumin for 12 h. Quantitative real-time POR reactions to assess ENPP2 were done with 100 ng of RNA for ENPP2 and β-actin (endogenous control). The amount of ENPP2 message was normalized to β-actin levels and reported as a relative value. Representative data of three independent experiments. **, P < 0.01, versus the control group (mock control cell line without curcumin treatment).
the multimodality approach using curcumin may lead to the development of an efficient antimetastatic agent without increased toxicity.

Discussion

Our study establishes a novel mechanism by which curcumin regulates integrin function. Specifically, we have shown that curcumin effectively inhibits cancer cell mobility and invasion toward a chemoattractant in a concentration-dependent manner by suppressing \( \alpha_6 \beta_4 \)-dependent Akt activation and expression of the cell motility-promoting factor ENPP2. Even at low doses, curcumin also acts cooperatively with other pharmacologic inhibitors against key signaling effectors of \( \alpha_6 \beta_4 \), such as c-Met and Akt. Considering the fact that \( \alpha_6 \beta_4 \) enhances migration and invasion of aggressive cancer cells, our data suggest a potential role of curcumin as an antimetastatic agent.

High doses (8 g/d) of curcumin can be delivered to patients with virtually no deleterious side effects and were found to generate a 1.77 ± 1.87 \( \mu \)mol/L average peak serum concentration in a phase I clinical trial (38). We found that even at the lower doses (2-5 \( \mu \)mol/L), curcumin effectively reduced cancer cell motility and invasion. Therefore, our finding is encouraging because the low concentrations are well within a range physiologically achievable in cancer patients. The multimodality approach using curcumin (Fig. 5) also suggests the possibility that combination of curcumin with other pharmacologic inhibitors could potentially create effective anticancer therapeutic cocktails in which the overall dose of each component could be reduced enough to lower the risk of side effects.

Whereas our data show that curcumin reduces the phosphorylation of the key tyrosine residue (Y1494) of the \( \beta_4 \)-integrin cytoplasmic tail, which is important for its function (28), the mechanism by which curcumin regulates \( \alpha_6 \beta_4 \) function remains to be determined. If curcumin inhibition of \( \alpha_6 \beta_4 \) is direct, it is likely that curcumin inhibits the kinase that phosphorylates Y1494 (identity currently unknown) or activates a phosphatase that dephosphorylates this residue. If the inhibition is indirect, curcumin may regulate the expression of some genes involved in the regulation of \( \alpha_6 \beta_4 \) activity. These possibilities are currently under investigation. Regardless of whether curcumin inhibition of \( \alpha_6 \beta_4 \) function is direct or indirect, its inhibitory effect on cancer cell motility seems to be more pronounced in the \( \alpha_6 \beta_4 \)-positive cancer cell lines (Fig. 1). \( \alpha_6 \beta_4 \)-negative cancer cell lines such as the MDA-MB-435 mock clone are far less motile and invasive compared with \( \alpha_6 \beta_4 \)-positive cancer cell lines (Fig. 1), and the effect of curcumin on their growth or motility was minimal. These...
results suggest the novel possibility that the presence of αβ3β4 may sensitize cancer cells to curcumin treatment. In other words, frequent up-regulation of αβ3β4 in cancer cells may explain why curcumin selectively targets cancer cells over normal cells. This possibility will be tested in our future studies.

Based on our findings, we conclude that curcumin may prove to be a potent antimigratory agent that potentially prevents the spread of the breast cancer from its primary origin to distant organs. This activity is associated with the successful inhibition of Akt, the downstream target of αβ3β4 integrin. Therefore, our findings suggest that curcumin could be used in novel anticancer therapeutics for breast cancer patients whose primary tumors overexpress αβ3β4 integrin.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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