Silibinin Suppresses EGFR Ligand-induced CD44 Expression through Inhibition of EGFR Activity in Breast Cancer Cells

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Abstract. CD44, the transmembrane receptor for hyaluronan, is implicated in tumor cell invasion and metastasis. The expression of CD44 and its variants is associated with poor prognosis in breast cancer. Here, we investigated the effect of silibinin (a polyphenolic flavonolignan of the herbal plant of Silybum marianum, milk thistle) on the epidermal growth factor (EGF) ligand-induced CD44 expression in human breast cancer cells. The levels of CD44 mRNA and protein expression were greatly increased by EGF and by TGF-α in SKBR3 and BT474 breast cancer cells. In contrast, EGFR ligand-induced CD44 expression was reduced by EGFR inhibitors, AG1478 and lapatinib, respectively. Interestingly, we observed that EGFR ligand-induced CD44 and matrix metalloproteinase-9 (MMP-9) expression was reduced by silibinin treatment in a dose-dependent manner. In addition, silibinin suppressed the EGF-induced phosphorylation of EGFR and extracellular signal-regulated kinase1/2 (ERK1/2), a downstream signaling molecule of EGFR. Therefore, we suggest that silibinin prevents the EGFR signaling pathway and may be used as an effective drug for the inhibition of metastasis of human breast cancer.

Breast cancer is one of the most common female malignancies in many industrialized countries and it comprises a remarkably heterogeneous group of diseases (1). Despite advances in the early detection of breast cancer and the advent of novel targeted therapies, there is still a high failure rate, mainly due to tumor invasion and metastasis (2, 3). Therefore, the search for new therapeutic agents and novel targets is very important to prevent and treat breast cancer.

Silibinin (Figure 1) is a polyphenolic flavonolignan of the herbal plant of Silybum marianum, milk thistle, and has a wide range of pharmacologic effects, such as antihepatotoxic and anticarcinogenic in a variety of tumor cells (4, 5). In a previous study, we also reported that 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 expression is inhibited by silibinin through inactivation of the RAF/MEK/ERK pathway (6), as well as augmenting the cell cycle arrest at the G2/M phase through the induction of p21 and the reduction of cyclin B1 in breast cancer cells (7). However, the mechanism underlying the inhibitory action of silibinin on EGF signaling and metastasis has not been completely elucidated. Here, we also studied the effect of silibinin on EGFR ligand-induced CD44 expression, which is a hallmark of metastasis in various cancer cells.

The CD44, an 85-90 kDa transmembrane glycoprotein, is a receptor of the extracellular matrix component hyaluronan (HA) (8). The standard form of CD44 is usually ubiquitously expressed on epithelial cells and lymphocytes, but inclusion of one or more of 10 variant exons produces tissue-specific multiple variant isoforms (CD44v2–v10) by alternate mRNA splicing (9). HA-bound CD44 triggers intracellular signaling that has been linked to diverse cellular functions, such as adhesion and invasion during tumor progression and metastasis (10). In particular, the splice variants of CD44v6 are associated with aggressiveness of cancer and correlate with poor prognosis in breast cancer (11).

In this study, we investigated the effect of silibinin on EGF signaling pathway and EGF ligand-induced CD44 expression in breast cancer cells.

Materials and Methods

Reagents. RPMI-1640 and the antibiotics used were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Thermo-Fisher Scientific (Waltham, MA, USA). Silibinin was purchased from Sigma (St. Louis, MO, USA).
AG1478 was purchased from Tocris (Ellisville, MO, USA). Lapatinib was purchased from Selleck Chemicals (Houston, TX, USA). The secondary horseradish peroxidase (HRP)-conjugated antibodies (mouse and rabbit) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). EGF and transforming growth factor (TGF)-α were purchased from R&D Systems (Minneapolis, MN, USA). The ECLplus reagents were from Amersham (Buckinghamshire, UK).

Cell culture and cell viability. The human breast cancer cell lines SKBR3 and BT474 were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Total cell numbers by silibinin were evaluated by Quick Cell Proliferation Assay Kit II (BioVision, Mountain View, CA, USA) according to the manufacturer’s protocol. Briefly, SKBR3 and BT474 human breast cancer cells (5×10⁴/well) were grown in a 96-well plate in 100 μl/well of culture media in the absence or presence of the indicated concentration of silibinin. After incubating the cells for 24 h, 10 μl WST cell proliferation reagent was added to each well. Viable cells were quantified photometrically at 480 nm.

Silibinin and chemical treatment. SKBR3 human breast cancer cells were maintained in culture medium without FBS for 24 h, and then the culture medium was replaced with fresh medium without FBS and the cells were further incubated with the indicated concentrations of silibinin for 24 h. In the drug treated experiments involving silibinin, AG1478, or lapatinib, the cells were pretreated with silibinin, AG1478, and lapatinib for 60 min prior to treatment with EGF or TGF-α, respectively, and then they were treated with EGF or TGF-α for 24 h.

Western blotting. SKBR3 breast cancer cell lysates were used in the immunoblot analysis for analyzing of protein expression. The proteins were boiled for 5 min in Laemmli sample buffer and then they were electrophoresed in 8% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes and the membranes were then blocked with 10% skim milk in tris buffered saline (TBS) with 0.01% Tween-20 (TBS/T) for 15 min. The blots were incubated with anti-t-EGFR, p-EGFR, CD44, p-ERK1/2, and β-actin antibodies in TBS/T buffer at 4°C overnight. The blots were washed 3 times in TBS/T and they were subsequently incubated with anti-rabbit peroxidase-conjugated antibody (1/2,000 dilution)
in TBS/T buffer. After 1 h incubation at room temperature (RT), the blots were washed three times in TBS/T and ECL plus reagents were used for development.

Real-time polymerase chain reaction (PCR). The total RNA was extracted from treated cells by using TRizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. Isolated RNA samples were then used for RT-PCR. Samples (1 μg of total RNA) were reverse-transcribed into cDNA in 20 μl reaction volumes using a first-strand cDNA synthesis kit for RT-PCR, according to the manufacturer’s instructions (MBI Fermentas, Hanover, MD, USA).

The gene expression was quantified by real-time PCR using a SensiMix SYBR Kit (Bioline Ltd., London, UK) and 100 ng of cDNA per reaction. The sequences of the primer sets used for this analysis were as follows: human CD44: forward, 5’- CCA AGA TGA TCA GCC ATT CTG G-3’; reverse, 5’-AAG ACA TCT ACC CCA GCA AC-3’, and β-actin as an internal control: forward, 5’-AAA CTG GAA CGG TGA AGG TG-3’; reverse, 5’-CTC AAG TTT GGG GAC AAA AA-3’. An annealing temperature of 60˚C was used for all of the primers. PCRs were performed in a standard 384-well plate format with an ABI 7900HT real-time PCR detection system. For data analysis, the raw threshold cycle (C_T) value was first normalized to the housekeeping gene for each sample to obtain the ΔC_T. The normalized ΔC_T was then calibrated to the control cell samples to give the ΔΔC_T.

Confocal microscopy. Human breast cancer SKBR3 cells grown on 4-well Lab-Tek chamber slides were allowed to adhere overnight, and then serum-starved for 24 h before 50 ng/ml EGF and/or silibinin treatments for 24 h. Cells were fixed for 20 min in 4% paraformaldehyde and then the fixed cells were dehydrated in ethanol. Cells were permeabilized in 0.5% Triton X-100 in PBS for 5 min at RT and were incubated at 4˚C overnight with anti-CD44 antibody (1:400 dilution, 156-3C, Cell Signaling Technology, Beverly, MA, USA) and then washed 3 times in PBS, slides were incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:500 dilution) for 60 min at RT. Cells were then washed and slides were mounted in Vectashield H-1200/DAPI mounting media (Vector Laboratories, Burlingame, CA, USA). Confocal images were analyzed using a LSM700 confocal laser-scanning microscope (Carl Zeiss, Germany).
Zymography. To analyze MMP-9 expression, zymography was performed using the 10% SDS-PAGE gels that had been cast in the presence of gelatin as described previously (12). Briefly, samples (100 μl) were lyophilized to dryness in a speed-vac concentrator and then were resuspended in loading buffer and these were run without prior denaturation. After electrophoresis, the gels were washed to remove the SDS and then they were incubated for 30 min at RT in renaturing buffer (50 mM Tris, 5 mM CaCl₂, 0.02% NaN₃, 1% Triton X-100). The gels were then incubated for 48 h at 37˚C in developing buffer [50 mM Tris-HCl (pH 7.8) 5 mM CaCl₂, 0.15 M NaCl and 1% Triton X-100]. The gels were subsequently stained with Coomassie Brilliant Blue G-250 and then they were destained in 30% methanol and 10% acetic acid to detect gelatinase secretion.

Statistical analysis. Statistical significance was determined using Student’s t-test. The results are presented as means±SEM. All quoted p-values are two-tailed and differences are considered significant for p-values <0.05.

Results

The basal level of CD44 is dose-dependently increased by EGF and by TGF-α treatment in breast cancer cells. To investigate the correlation between EGFR and CD44 expression, we treated the SKBR3 and BT474 human breast cancer cells with the indicated concentrations of EGF or TGF-α for 24 h. Our results showed that the basal level of CD44 mRNA expression was dose-dependently increased by EGF and by TGF-α in SKBR3 cells (Figure 2A). The level of CD44 mRNA expression was significantly increased to 32.4±4.8-fold and 44.9±5.1-fold that of the control level by 50 ng/ml EGF or TGF-α treatment, respectively (Figure 2A). In addition, we also observed the induction of CD44 mRNA by EGF and TGF-α in BT474 cells (Figure 2B). As shown in Figure 2C, the level of CD44 protein expression was also significantly increased by EGF in SKBR3...
cells. Based on these results, we demonstrated that the expression of $CD44^{\text{mRNA}}$ and protein appears to be mediated through EGFR-dependent pathway in breast cancer cells.

**EGF- and TGF-α-induced $CD44^{\text{mRNA}}$ expression is reduced by EGFR inhibitors in SKBR3 breast cancer cells.** To verify the effect of EGFR inhibitors on EGF- and TGF-α-induced $CD44^{\text{mRNA}}$ expression in SKBR3 cells, we pretreated them with 10 μM AG1478 and 2 μM lapatinib, respectively, and then treated them with EGF or TGF-α. After 24 h, we harvested the cell lysates and culture media for assessing the expression of $CD44^{\text{mRNA}}$ and protein, respectively. Both EGF and TGF-α significantly increased the level of $CD44^{\text{mRNA}}$ expression (Figure 3A and B). However, EGF- or TGF-α-induced $CD44^{\text{mRNA}}$ expression was reduced by EGFR inhibitors (Figure 3A and B). $CD44^{\text{mRNA}}$ expression was significantly increased to 15.9±0.45-fold and 8.0±1.1-fold that of the control level by EGF and TGF-α, respectively (Figure 3A and B). In contrast, both EGF- and TGF-α-induced $CD44^{\text{mRNA}}$ expression was reduced to 1.4±0.1-fold and 2.95±0.15-fold that of the control level by EGFR inhibitor AG1478 respectively (Figure 3A and B). Therefore, we demonstrated that EGFR ligands/EGFR signaling pathway directly regulates the level of $CD44^{\text{mRNA}}$ and protein expression.

**EGF- and TGF-α-induced $CD44^{\text{mRNA}}$ expression is reduced by silibinin of SKBR3 breast cancer cells.** To test the cytotoxicity of silibinin on breast cancer cells, we treated the SKBR3 and BT474 human breast cancer cells with the indicated concentrations of silibinin for 24 h. As shown in Figure 4A, the viability of SKBR3 and BT474 breast cancer cells did not depend on the concentration of silibinin. Next, we examined whether silibinin is involved in the EGF ligand-induced $CD44^{\text{mRNA}}$ expression. After pretreatment with silibinin for 60 min, the cells were treated with EGF or TGF-α for 24 h. Both EGF- and TGF-α-induced $CD44^{\text{mRNA}}$ expression was reduced by silibinin in a dose-dependent manner (Figure 4B and C). EGF-induced $CD44^{\text{mRNA}}$ expression was reduced to 4.9±0.5-fold and 3.3±0.1-fold that of the control level by 25 and 50 μM silibinin treatment, respectively (Figure 4B). In addition, TGF-α-induced $CD44^{\text{mRNA}}$ was also reduced to 2.8±0.3-fold and 2.1±0.5-fold that of the control level by 25 and 50 μM silibinin treatment, respectively (Figure 4C).
Confocal analysis was carried out next to determine the expression and distribution of CD44 protein in SKBR3 breast cancer cells. Our results showed that CD44 is predominantly distributed at the plasma membrane (Figure 5A). Furthermore, the level of CD44 protein expression was significantly increased by EGF whereas EGF-induced CD44 expression was reduced by silibinin (Figure 5A). This suggests that silibinin inhibits the EGF/EGFR signaling pathway in breast cancer cells.

EGF-induced CD44 expression and the phosphorylation of EGFR and ERK are suppressed by silibinin treatment of SKBR3 breast cancer cells. Finally, we investigated the effect of silibinin on EGF/EGFR signaling pathway in SKBR3 breast cancer cells. Cells were pretreated with the indicated concentration of silibinin for 60 min and then treated with EGF for 24 h. As shown in Figure 5B, the phosphorylation of EGFR and downstream signaling molecule ERK1/2 was increased by EGF treatment, while EGF-induced EGFR and ERK1/2 phosphorylation were dose-dependently reduced by silibinin.

In a previous study, we reported that EGF-induced MMP-9 expression was mediated through JAK3/ERK-dependent pathway in SKBR3 breast cancer cells (12). MMP-9 plays an important role in cancer cell invasion and metastasis through the degradation of all the extracellular matrix (ECM) components (13, 14). Therefore, we examined the effect of silibinin on EGF-induced MMP-9 expression. EGF-induced MMP-9 expression was reduced by silibinin in a dose-dependent manner (Figure 5B). Based on these results, we demonstrated that silibinin prevents EGF-induced CD44 expression, as well as MMP-9 expression through the inhibition of the EGF/EGFR signaling pathway in breast cancer cells.

Discussion

CD44 is widely distributed in a variety of cells and plays a major role in multiple physiological processes, including cell cell adhesion and tumor metastasis (15, 16). In addition, HA-bound CD44 correlated with tumor cell invasiveness and enhanced tumor cell migration during metastasis (17). The overexpression of CD44v6, one splice variants, leads to augmented tumor formation and lymph node metastasis of lymphoma cells (18, 19). Antibody-mediated CD44 crosslinking leads to an enhanced level and relocation of MMP-9 in the membrane of human breast tumor cells, accompanied by increased tumor invasion and metastasis (17). Although we did not directly investigate the interaction of CD44 with MMP-9, EGF ligand-induced CD44 and MMP-9 expressions were reduced by silibinin. Therefore, we demonstrated that silibinin may act as a potential antimetastatic drug through the suppression of CD44 expression in breast cancer cells.

Overexpressed EGFR on tumor cell surface is associated with tumor aggressiveness, and the activation of EGFR upon binding of its ligands such as EGF and TGF-α, modulates cell adhesion, migration, and differentiation under physiologic and pathologic conditions (20). EGF regulates cellular interactions with ECM components such as hyaluronate, by modulating CD44 expression, and was found to enhance the murine fibroblast NR6 cell attachment to the ECM (21). In addition, Lamb et al. reported that increased expression of CD44 is dependent upon the transcriptional activity of activator protein-1 (AP-1) in FOS- and EGF-transformed cells and plays an important role in fibroblast invasion (22). Consistent with these reports, we also found that the level of CD44 expression is significantly increased by EGF and TGF-α, whereas EGF ligand-induced CD44 expression is completely suppressed by EGF inhibitors AG1478 and lapatinib. Our results showed that silibinin acts as a powerful inhibitor of the EGFR signaling pathway in breast cancer cells.

The MMPs are regarded as major critical molecules that assist tumor cells during cancer cell invasion and metastasis through the degradation of ECM components (13, 14). Thus, numerous studies focused on the inhibition of MMPs by synthetic and natural inhibitors for treatment of tumor cell invasion and metastasis (23, 24). In the present study, we observed that EGF-induced MMP-9 expression was dose-dependently reduced by silibinin. Therefore, we believe that silibinin may show great promise in a trial of patients with breast cancer through the inhibition of metastasis-related genes such as that for MMP-9.

In summary, we demonstrated that EGFR ligands, EGF and TGF-α significantly augment the expression level of CD44 mRNA and protein through the activation of EGFR pathway in breast cancer cells. Interestingly, silibinin prevents EGF-induced the phosphorylation of EGFR and then results in suppression of induction of CD44 expression by EGFR ligands. In addition, silibinin also suppressed EGF-induced expression of MMP-9, which is a key enzyme for the degradation of ECM proteins such as type I and IV collagen during tumor invasion and metastasis. Therefore, we suggest that silibinin may be used as a powerful drug for the inhibition of tumor invasion and metastasis of human breast cancer through the suppression of the CD44 and MMP-9 expressions.

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References

Kim et al: Inhibition of EGFR Activity by Silibinin

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